'Determination of an interaction between DNA repair proteins MLH1 and sMBD4 and aspirin regulation of DNA repair gene and protein expression in colorectal cancer'

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Signature.....

Date.....

Abstract

The base excision repair protein, MBD4 (also known as MED1) is known to be transcriptionally coupled to a mismatch repair protein MLH1. To date the significance of this coupling has not been elucidated and the significance of MBD4 within the mismatch repair system and apoptotic pathway is still being understood. Recently a novel alternatively spliced form of MBD4 has been identified and termed sMBD4. To date the significance of sMBD4 is unknown. MBD4 and sMBD4 share a common glycosylase domain and this is the domain through which MBD4 is reported to interact with MLH1. It was the aim of this study to determine if sMBD4 was also a binding partner of MLH1 to help elucidate a potential role of sMBD4 and to further characterise the binding domain between MLH1 and MBD4. Recombinant proteins were utilised in binding assays however, a specific protein – protein interaction could not be determined.

Regular aspirin intake is associated with a reduction in the incidence of colorectal cancer. Aspirin has been shown to be cytotoxic to colorectal cancer cells *in vitro*. The molecular basis for this cytotoxicity is controversial, with a number of competing hypotheses in circulation. One suggestion is that the protective effect is related to the induction of DNA mismatch repair (MMR) proteins in DNA MMR proficient cells. As MBD4 has previously been suggested to be coupled to MLH1 expression by a post-translational mechanism the cytotoxicy of aspirin in relation to MBD4 expression was examined. This study reports that aspirin does not up-regulate *MBD4* gene transcription *in vitro* in the DNA mismatch repair proficient/p53 mutant colorectal cancer cell line SW480. However, *MBD4* gene transcription was up-regulated upon treatment with the aspirin precursor, salicylic acid.

The suggested involvement of the DNA repair proteins in the mechanism of action of aspirin promoted the investigation into the expression of DNA damage signalling pathways genes upon aspirin exposure. This study utilised a commercially available PCR array to analyse the expression of 84 DNA damage signalling genes in the SW480 colorectal cancer cell line upon aspirin treatment. It is reported that treatment of the SW480 cell line with aspirin caused changes in mRNA expression of several key genes involved in DNA damage signalling including a significant down-regulation in expression of the genes encoding *ATR*, *BRCA1* and *MAPK12* and increases in the expression of *XRCC3* and *GADD45α* genes. Regulation of these genes could potentially have profound effects on colorectal cancer cells and may play a role in the observed chemo-protective effect of aspirin *in vivo*.

Further to this, protein expression was analysed to determine if correlation could be established with the changes in mRNA expression observed. Although a correlation was not seen between transcript and protein levels of ATR, BRCA1 and GADD45α, an increase in XRCC3 protein expression upon aspirin treatment in SW480 cells was observed by immunoblotting, immunofluorescence and immunohistochemical analysis. This study indicates that alterations in gene expression seen in microarray studies need to be verified at the protein level. Furthermore, this study reports the novel discovery of XRCC3 gene and protein expression being susceptible to exposure to the non-steroidal anti-inflammatory drug, aspirin.

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Abbreviations

5-FU	5-Fluorouracil
9-1-1	Rad9 – Hus1 – Rad1 complex
18S	18S Ribosomal RNA
A	Adenine
ADP	Adenosine 5’-diphosphate
ADP	Analytical digital photomicroscopy
AMP	Adenosine 5’-monophophate
AMV	Avian Myeloblastosis Virus Reverse transcriptase
ANOVA	Analysis of variance
AP	Apurinic/apyrimidinic
APE	Apurinic/apyrimidinic endonuclease
APS	Ammounium persulphate
ATM	Ataxia telangiectasia mutated
ATP	Adenosine 5’-triphosphate
ATR	Ataxia telangiectasia and Rad3 related
ATRIP	ATR-interacting protein
BASC	BRCA1 associated genome surveillance complex
BER	Base Excision Repair
BLM	Bloom’s syndrome protein
BRCA1	Breast Cancer Associated gene 1
BSA	Bovine Serum Albumin
C	Cytosine

cDNA	Complementary DNA
CIU	5-chlorouracil
CO_2	Carbon dioxide
COX	Cyclo-oxygenase
COX-1	Cyclo-oxygenase-1
COX-2	Cyclo-oxygenase-2
CpG	Cytosine-phosphate-Guanine
$cPLA_2$	Cytosolic phospholipase A_2
DAPI	4',6 diamidino-2-phenylindole
DMEM	Dulbecco's Modified Eagle's Medium
DMSO	Dimethyl Sulphoxide
DNA	Deoxyribonucleic acid
DNMT	DNA methyltransferase
dNTP	Deoxyribonucleotide triphosphate
DTT	Dithiothreitol
E. coli	*Escherichia coli*
ECACC	European Collection of Animal and Cell Culture
EDTA	Ethylenediaminetetra acetic acid
EXO1	Exonuclease 1
FADD	Fas-associated death domain protein
FAP	Familial adenomatous polyposis
FCS	Foetal calf serum
G	Guanine

GABA	Gamma-aminobutyric acid
GADD45	Growth arrest and DNA-damge-inducible protein
GADD45α	Growth arrest and DNA-damage-inducible protein alpha
GADD45β	Growth arrest and DNA-damage-inducible protein beta
GADD45λ	Growth arrest and DNA-damage-inducible protein gamma
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GST	Glutathione S-transferase
HCl	Hydrochloric acid
HCT116	Human colon cancer cell line
His	Histidine
HNPCC	Hereditary non-polyposis colorectal cancer
HPS	Hyperplastic polyposis syndrome
HRP	Horse Radish Peroxidase
HT29	Human colon cancer cell line Grade II
ICAM-1	intracellular adhesion molecule 1
IPTG	Isopropyl β-D-1-thiogalactopyranoside
IκB	I kappa B
IκBα	I Kappa B alpha
IKK	I kappa B Kinase
IKKβ	I kappa B Kinase beta
LB	Luria Broth
M	average expression ability
m^5C	5 - methylcytosine

MAPK	Mitogen activated protein kinase
MAPK12	Mitogen activated protein kinase 12
MAX	MYC associated factor X
MBD	Methyl Binding Domain
MBD1	Methyl Binding Domain protein 1
MBD2	Methyl Binding Domain protein 2
MBD3	Methyl Binding Domain protein 3
MBD4	Methyl Binding Domain Protein 4
MCF7	human breast cancer cell line
MDA231-MB	human breast cancer cell line
MECP2	Methyl-CpG-binding protein 2
MED1	Methyl-CpG Binding Endonuclease I
MEM	Modified Eagles Medium
Mg^{2+}	Magnesium ions
MgCl	Magnesium Chloride
MIC-1	Macrophage inhibitory cytokine-1
MLH1	MutL homolog 1
MMR	Mismatch Repair
MTT	3-(4,5-dimethylthazol-2-yl)-2,5-diphenyltetrazolium bromide
MRE11	Meitotic recombination 11
mRNA	Messenger RNA
MSI	Microsatellite instability
MSH2	MutS homologue 2

MSH6	MutS homologue 6
NaCl	Sodium Chloride
NAG-1	NSAID-activated gene
NaOH	Sodium Hydroxide
NBS1	Nijmegen breakage syndrome 1
NDR1	non-race specific disease resistance 1
NFκB	Nuclear Factor – Kappa B
NPR1	Non-expressor of PR genes 1
NSAID	Non-steroidal anti-inflammatory drug
OD	Optical density
P38 MAPK	p38 isoform of mitogen-activated protein kinase
PBS	Phosphate buffered saline
PCNA	Proliferating cell nuclear antigen
PCR	Polymerase chain reaction
PDF	Prostate-derived factor
PGE_2	Prostaglandin E_2
PIKKs	Phosphoinositide 3-kinase related protein kinases
PMS1	Post-meiotic segregation increased 1
PMS2	Post-meiotic segregation increased 2
PMSF	Phenylmethanesulfonylfluoride
PTGF-β	Placental transforming growth factor beta
PVDF	Polyvinylidene fluoride
RER^+	Replication Error Positive

RIPA	Radio-immune precipitation assay
RPA	Replication Protein A
RPC	Replication Factor C
RNA	Ribonucleic acid
RNAi	Ribonucleic acid interference
RT-PCR	Reverse transcription-polymerase chain reaction
SDS	Sodium dodecyl sulphate
SDS-PAGE	Sodium dodecyl sulphate-polyacrylamide gel electrophoresis
SEM	Standard error of the mean
SPR	Surface Plasmon resonance
ssDNA	single stranded DNA
SW480	Human colon cancer line
T	Thymine
TBE	Tris-Borate-EDTA
TBS-T	Tris-Buffered Saline Tween 20
TEMED	N,N,N',N'-Tetramethylethylenediamine
TGF-β	Transforming growth factor beta
TNF-α	Tumour necrosis factor alpha
TRAIL	Tumour necrosis factor-related apoptosis-inducing ligand-induced
Tris	2-amino-(hydroxymethyl) propane-1,3-diol
U373MG	Human astrocytoma cell line
UDG	Uracil DNA glycosylase
UV	ultraviolet

V	Pairwise variation
XRCC3	X-ray repair complementing defective repair in Chinese hamster cells 3

Units

^{o}C	Degrees Celsius
µ	micro (10^{-6})
m	milli (10^{-3})
n	nano (10^{-9})
M	Molar
bp	base pair
l	Litre
g	gram
kDa	kilo Dalton
rpm	revolutions per minute
U	Unit
v/v	volume per volume
w/v	weight per volume

Chapter 1

Introduction

1 Introduction

1.1 Colorectal cancer

More than 940,000 colorectal cancer cases occur annually worldwide whilst approximately half a million patients die each year (Boyle and Levin, 2008). Approximately 2 - 5% of cases of colorectal cancer are due to a genetic predisposition of which the most common is hereditary non-polyposis colorectal cancer (HNPCC) also known as Lynch Syndrome (Mecklin, 2008). The term Lynch Syndrome is given when a mutation in a DNA mismatch repair (MMR) gene is confirmed and the term HNPCC is given to patients who display the clinical features but have no confirmed genetic mutation (Mecklin, 2008).

1.1.1 The genetics of hereditary colon cancer

HNPCC is an autosomal dominant disorder where there are heterozygous germline mutations in genes involved in the DNA MMR system such as MutS homologue 2 (*MSH2*), MutL homologue 1 (*MLH1*), MutS homologue 6 (*MSH6*), post-meiotic segregation increased 2 (*PMS2*) and post-meiotic segregation increased 1 (*PMS1*) (Narayan and Roy, 2003; Buermeyer *et al.,* 1999; Bocker *et al.,* 1999; Plaschke *et al.,* 2004; Nicolaides *et al.,* 1998). However, somatic mutations can also occur. As the MMR system is compromised, defects in DNA replication can occur leading to cancer causing mutations. *MLH1* and *MSH2* mutations are the most common with abnormalities in these genes found in more than 90% of HNPCC mutation carriers (Papadopoulos and Lindblom, 1997; de la Chapelle, 2005; Abdel-Rahman *et al.,* 2006).

A consequence of deficient MMR is the accumulation of mutations in repetitive nucleotide regions a phenomenon termed microsatellite instability (MSI) (Gologan and Sepulveda, 2005; Buermeyer *et al.,* 1999). Microsatellites are simple tandem nucleotide sequence repeats of 1 – 6 base pairs in the genome. Changes in the number of the repeat units due to defective MMR are mutagenic and as such the MSI phenotype or replication error positive (RER^+) is considered a marker of MMR deficiency (Spampinato *et al.,* 2009). HNPCC is therefore closely linked with MSI and approximately 15% of sporadic colorectal tumours also demonstrate MSI (Aaltonen *et al.,* 1993; Thibodeau *et al.,* 1998) suggested to be linked to transcriptional silencing of *MLH1* (Herman *et al.,* 1998).

1.1.2 Diagnostic criteria

The diagnostic criteria for HNPCC have been amended over the years in light of new information discovered about the syndrome. In 1991 the 'Amsterdam criteria I' guidelines were developed to aid diagnosis of HNPCC. However, at the time the genetic basis for HNPCC was unknown and as extra-colonic cancers, i.e., malignancies other than colorectal cancer occurring in patients with HNPCC such as endometrial, urothelial and small bowel carcinomas became apparent, the Amsterdam criteria I list was found to be too stringent and was therefore revised to the 'Amsterdam Criteria II' (Table 1.1). As the genetic mechanisms of HNPCC came to light, in particular the discovery of MSI, the Bethesda Criteria and the subsequent revised Bethesda Criteria were introduced (Table 1.1) (Jass, 2006; Rustgi, 2007).

Table 1.1 Comparison of the Amsterdam Criteria I and II and the original Bethesda and revised Bethesda Criteria for diagnosis of HNPCC. (Table adapted from Rustgi, 2007)

AMSTERDAM CRITERIA I	At least three relatives with **colorectal cancer** and the following: *one should be a first-degree relative of the other two *at least two consecutive generations should be affected *at least one case of colorectal cancer should be before age 50 Familial adenomatous polyposis (FAP) should be excluded Verification of tumours' histopathology
AMSTERDAM CRITERIA II	At least three relatives with **HNPCC-related cancers** and the following: *one should be a first-degree relative of the other two *at least two consecutive generations should be affected *at least one case of HNPCC-related cancer should be before age 50 Familial adenomatous polyposis (FAP) should be excluded Verification of tumours' histopathology
ORIGINAL BETHESDA CRITERIA	Individuals with cancer in families that meet the Amsterdam criteria Individuals with two HNPCC-related cancers, including synchronous and metachronous colorectal cancers or associated extra-colonic cancers (endometrial, ovarian, gastric, heptobiliary or small bowel cancer or transitional cell carcinoma of the renal pelvis or ureter) Individuals with colorectal cancer and a first-degree relative with colorectal cancer and/or HNPCC-related extra-colonic cancer and/or a colorectal adenoma; one of the cancers diagnosed at <50 years of age, and the adenoma diagnosed at < 40 years of age Individuals with colorectal cancer or endometrial cancer diagnosed at < 50 years of age Individuals with right-sided colorectal cancer with an undifferentiated pattern (solid/cribriform) on histopathology diagnosed at < 50 years of age Individuals with signet-ring cell-type colorectal cancer diagnosed at < 50 years of age Individuals with adenomas diagnosed at < 40 years of age
REVISED BETHESDA GUIDELINES	Colorectal cancer diagnosed in a patient who is < 50 years of age Presence of synchronous or metachronous colorectal or other HNPCC - associated tumours (colorectal, endometrial, stomach, ovarian, pancreas, ureter and renal pelvis, biliary tract, small bowel, brain and sebaceous gland adenomas and keratoacanthomas), regardless of age Colorectal cancer with the MSI-high histology (presence of tumour infiltrating lymphocytes, Crohn's-like lymphocytic reaction, mucinous/signet-ring differentiation, or medullary growth pattern) diagnosed in a patient who is < 60 years of age Colorectal cancer diagnosed in one or more first-degree relatives with an HNPCC-related tumour, with one of the cancers being diagnosed under age 50 years Colorectal cancer diagnosed in two or more first- or second degree relatives with HNPCC-related tumours, regardless of age

1.2 DNA mismatch repair (MMR)

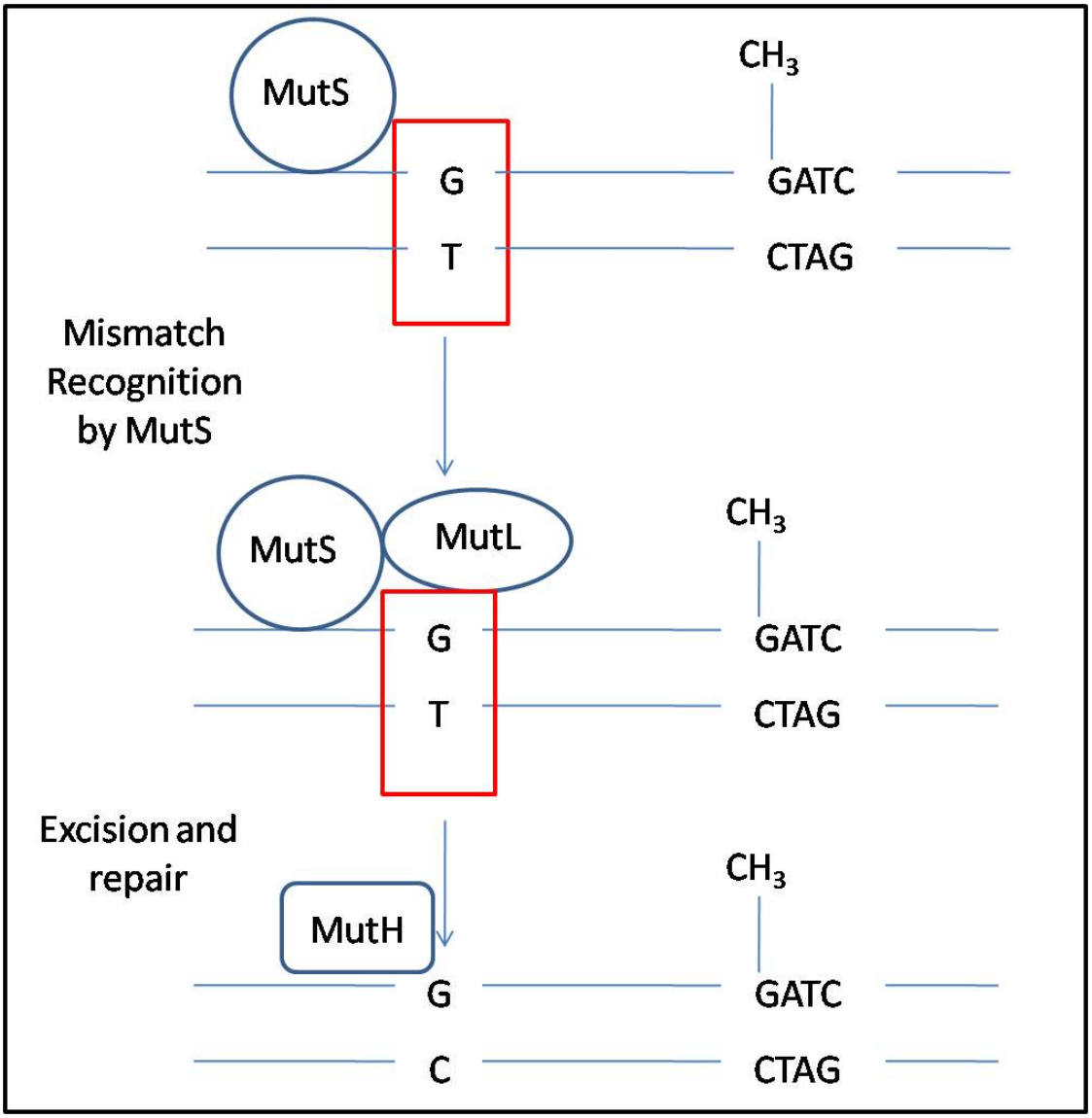
HNPCC is caused by mutations of MMR genes and as such is the consequence of defects in the MMR system (Lucci-Cordisco *et al.,* 2003; Jiricny, 1998; Zabkiewicz and Clarke, 2004; Marra and Schar, 1999). Defects in DNA replication can cause mutations which lead to the development of cancer (Jiriciny, 1998; Marra and Schar, 1999). The maintenance of genomic integrity is therefore essential to prevent DNA damage leading to malignant cells. The MMR system plays an essential role in identifying and correcting any replication errors and any additional errors which arise through physical or chemical damage. These errors may be base-base mismatches, short insertions/deletions and heteroduplexes, which can occur during DNA replication and recombination. The DNA MMR system therefore maintains genomic integrity and stability and in essence provides a tumour suppressor function. The abundance of literature in this field clearly represents the critical importance of this system with interest being fuelled by its link to colorectal cancer.

The essential nature of the MMR system to protect genomic integrity is best illustrated by investigating the underlying pathway steps; (i) DNA lesion recognition, (ii) identification and discrimination of the newly synthesized DNA strand, which carries the replication error, (iii) excision of the damaged DNA section and (iv) DNA resynthesis and ligation. These steps have been conserved throughout evolution, although modifications have resulted in a more complex system of proteins in mammalian cells than in prokaryotes (Spampinato *et al.,* 2009).

1.2.1 DNA mismatch repair in Prokaryotes

Early studies on MMR utilised *Escherichia coli* (*E. coli*) as a model and the MMR system in prokaryotes is now well defined. In *E. coli*, eight enzymes are required for MMR; MutL, MutS, MutH, DNA helicase II, single stranded DNA (ssDNA) binding protein, DNA polymerase III holoenzyme, an exonuclease and DNA ligase. Additionally the co-factors adenosine 5'-triphosphate (ATP), deoxynucleoside-5'-triphosphates (dNTPs), Mg^{2+} and the ligase co-factor NAD^{+} are required (Grilley *et al.,* 1990; Lahue *et al.,* 1989; Robinson and Brown, 2003). The DNA mismatch is recognised by the MutS homodimer which binds to the mismatch causing DNA loop formation (Buermeyer *et al.,* 1999). The MutL homodimer then physically interacts with MutS in the presence of ATP forming a complex and subsequently activates MutH (Robinson and Brown, 2003). MutH plays a key role in strand discrimination. Adenine methylation on the template strand allows the endonuclease MutH to recognise the hemimethylated site and hence strand discrimination. Subsequently, the site of the mismatch is nicked and excision is carried out along the shortest direction of the daughter strand followed by resynthesis. In *E. coli* there are either 5' – 3' (Exo VII and RecJ) or 3' – 5' (Exo 1, Exo VII and Exo X) exonucleases. The DNA polymerase III holoenzyme resynthesises the DNA strand which is then ligated to the pre-existing strand by DNA ligase (Krokan *et al.,* 2000; Jacob and Praz, 2002; Buermeyer *et al.,* 1999; Bowers *et al.,* 2000; Blackwell *et al.,* 2001; Drummond and Bellacosa, 2001; Spampinato *et al.,* 2009).

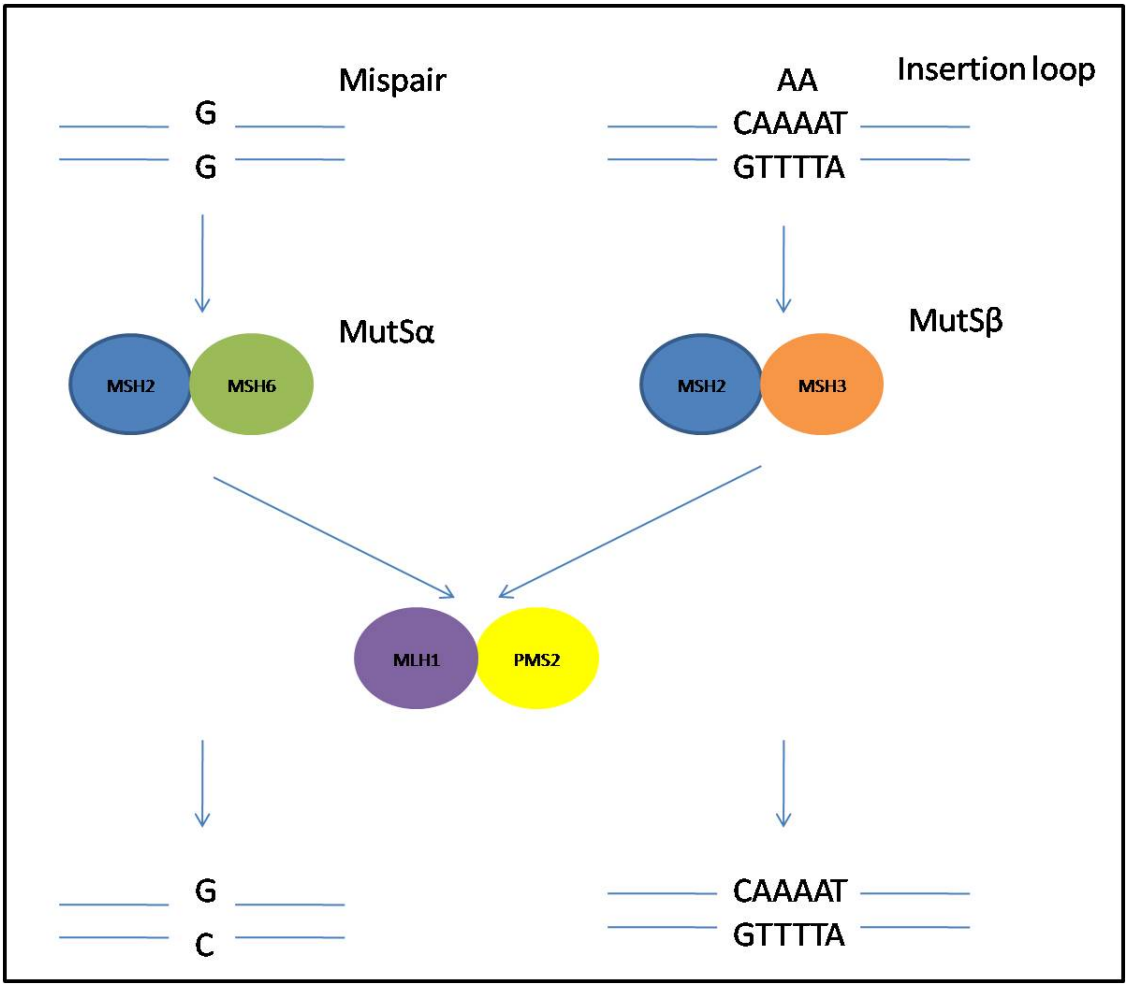
Figure 1.1 The DNA mismatch repair system of *Escherichia coli.* Schematic overview of the mismatch repair mechanism in *E. coli*. MutS recognises and binds to a DNA mismatch. MutL then binds to MutS and recruits MutH. Excision of the mismatch then occurs and subsequent resynthesis then follows (Figure adapted from Robinson and Brown, 2003).



1.2.2 DNA mismatch repair in mammalian cells

The central pathway of the MMR system in mammalian cells is similar to that of prokaryotes with homologues of MutS and MutL involved. Homologues of MutS are MSH2, MSH3 and MSH6 and homologues of MutL are MLH1 and PMS2 (Bowers *et al.,* 2000; Irving and Hall, 2001). In comparison to the MutS homodimer in *E. coli*, DNA damage in eukaryotes is recognised by heterodimer MutS homologues; either the MutSα homologue MSH2-MSH6 which recognises base-base mismatches (primarily G:T mismatches) and short insertion/deletion loops or MutSβ homologue MSH2-MSH3 which recognises larger insertion/deletion loops. Similarly, just as in *E. coli* where the MutL homodimer interacts with MutS, in eukaryotes the MutL homologue heterodimer MLH1-PMS2 interacts with the MutS homologue. Strand discrimination in eukaryotes is due to the leading and lagging strand discontinuities associated with DNA replication. It is also considered that proliferating cell nuclear antigen (PCNA) may be involved in strand discrimination by loading towards the 3' end of the newly synthesised strand and therefore assisting in strand specificity. Strand excision and resynthesis is essentially the same in prokaryotes and mammalian cells with the exception that there is no evidence of helicase activity in mammalian cells. Whilst in *E. coli* there are many exonucleases involved, in mammalian cells only the 5' – 3' exonuclease (Exo1) has been identified. In addition, in mammalian cells PCNA and replication factor C (RPC) are thought to be involved in bidirectional excision.

Figure 1.2 The DNA mismatch repair system in mammalian cells. Mismatched bases are recognised by the MutSα homologue MSH2-MSH6 and short insertion/deletion loops are recognised by the MutSβ homologue MSH2-MSH3. The MutS homologues interact with the MutL homologue heterodimer MLH1-PMS2. (Figure adapted from Wheeler *et al.,* 2000).



1.3 Colorectal Cancer and Aspirin

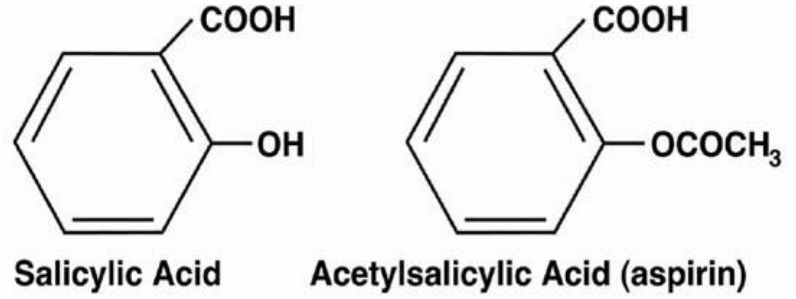
There is now abundant evidence that regular ingestion of aspirin and other non-steroidal anti-inflammatory drugs (NSAIDs) primarily known for their anti-inflammatory properties, can promote colorectal tumour regression and reduce the relative risk of developing colorectal cancer (Thun *et al.,* 1991; Giovannucci *et al.,* 1994; Schreinemachers and Everson, 1994; Sansom *et al.,* 2001; Thun *et al.,* 2002; Baron *et al.,* 2003; Imperiale, 2003; Sandler *et al.,* 2003). This is supported by randomised clinical trials (Baron *et al.,* 2003; Sandler *et al.,* 2003). There is also substantive evidence that aspirin is specifically cytotoxic against colorectal cancer cells cultured *in vitro* (Smith *et al.,* 2000) and in animal models aspirin can inhibit cancers induced by carcinogens such as 1,2-dimethylhydrazine (Barnes and Lee, 1999). In addition, evidence also suggests that aspirin reduces the incidence of recurrent adenomas, indicating it may be beneficial in preventing adenoma to carcinoma transformation (Baron *et al.,* 2003; Sandler *et al.,* 2003).

1.3.1 A Brief history of aspirin

Aspirin is the commercial name for acetylsalicylic acid and is one of the most widely known and used drugs currently available. Aspirin was first synthesized in 1897 by the scientist Felix Hoffmann, although there is controversy as to who credit should be given for the discovery of the drug (Sneader, 2000). Although first synthesised over 100 years ago, the story of aspirin dates back to the ancient times of Hippocrates about 2400 years ago. It was noted that willow bark extract, a natural source of salicylate, was effective against pain relief and also had antipyretic and anti-inflammatory effects. Although in this time there was no scientific validation or indeed understanding of how and why willow bark had these effects, due to its obvious clinical effectiveness against fever and inflammation, its use was continued. In the 18th and 19th centuries, advances in chemical technology meant that the compounds of willow bark could be characterised and it was in the 1820s that 'salicin' (derived from the Latin word for 'willow') was first identified as the active ingredient in willow bark. This purified salicin was prescribed to treat rheumatism. Salicin was then chemically modified to create 'salicylic acid' in the 1870s which, as well as maintaining the clinical properties of salicin, was also found to be a useful antiseptic. Indeed throughout the early 18th century, both salicin and salicylic acid were prescribed for fever, pain and rheumatic conditions. However, it was later observed that salicylic acid induced gastric irritation which was found to subside with co-administration of sodium bicarbonate. Also the

high doses of salicylic acid prescribed for conditions resulted in unwanted side effects such as nausea, vomiting and other gastrointestinal symptoms including bleeding and therefore interest was sparked into producing a safer salicylate. In 1887, Hoffmann modified salicylic acid to create acetylsalicylic acid, now known as aspirin, and was commercially marketed by the Bayer company (Figure 1.3). The name 'aspirin' was given for commercial purposes as it was considered easier to pronounce than 'acetylsalicylic acid'. The name is derived from 'spir' from *Spiraea ulmaria,* the plant from which salicylic acid was first extracted, with the suffix '-in' and an 'A' to designate the acetyl (Cheng, 2007). As aspirin was found to be better tolerated by the gastrointestinal tract than salicylic acid it became widely used (Rainsford, 2004).

Figure 1.3. The structures of salicylic acid and aspirin. Salicylic acid is an aspirin precursor; however its structure differs from aspirin as it does not contain the acetyl group found in aspirin. Aspirin retains the carboxyl group and has a substitution in the hydroxyl group (Image taken from Miner and Hoffhines, 2007).



1.3.2 Mechanisms of action of aspirin

Although studies and data have now provided evidence that regular use of aspirin based medication can reduce the risk of colorectal cancer, i.e., the use of aspirin is inversely associated with the risk of colorectal cancer (Rosenberg *et al.,* 1991; Thun *et al.,* 1991; Muscat *et al.,* 1994), the molecular basis for the anti-proliferative effect of aspirin on colorectal cancer cells is still largely unknown and controversial (and has been recently reviewed, Watson, 2006), with a range of theories in circulation which are discussed in further detail in the following sections below.

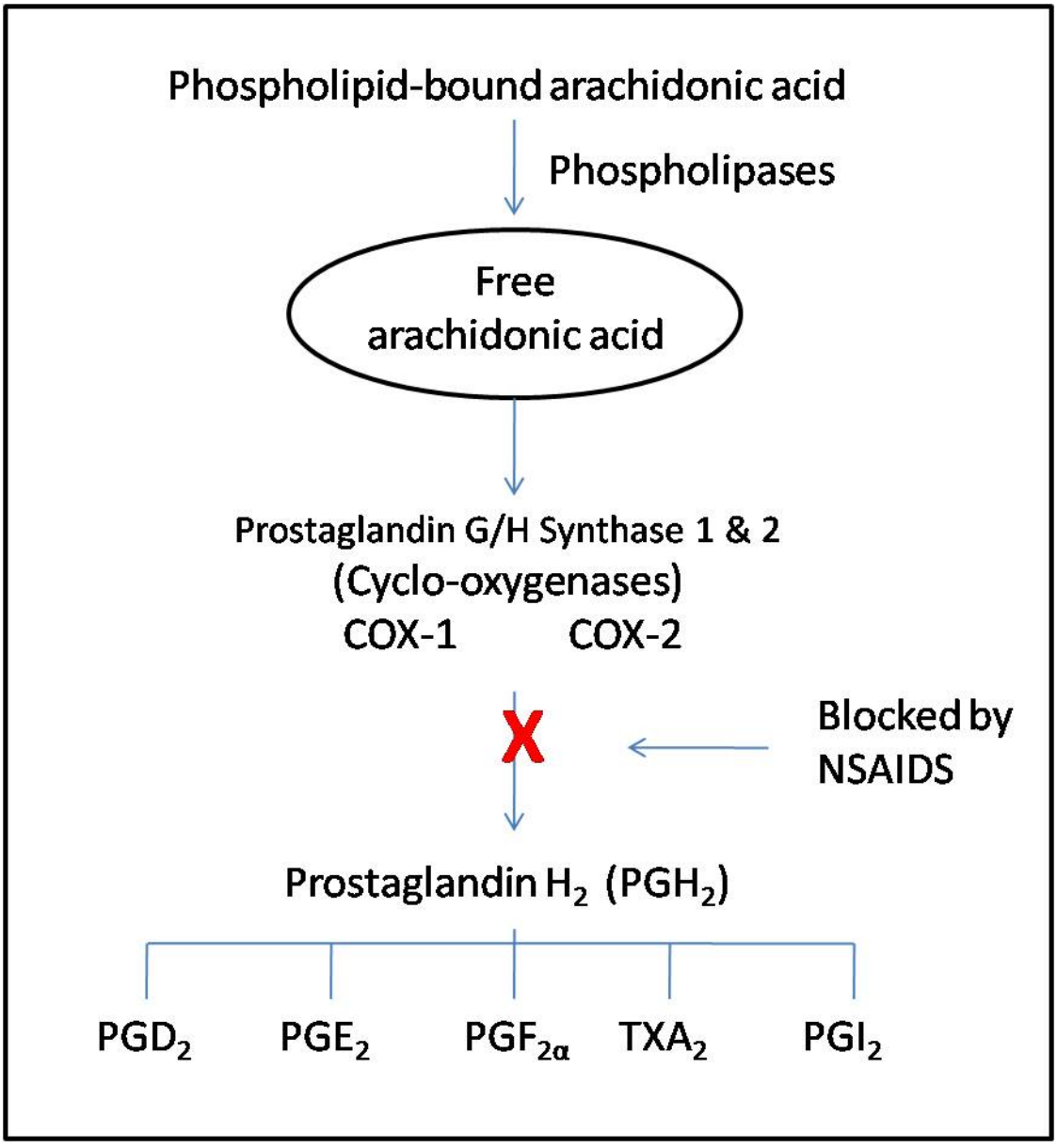
1.3.2.1 Cyclo-oxygenase (COX) inhibition

The main mechanism proposed for the chemoprotective effect of NSAIDs is via the inhibition of cyclo-oxygenase (COX) (Smith *et al.,* 2000; Ferrandez *et al.,* 2003). The key role of the COX enzyme is to metabolise arachidonic acid to the prostaglandins which are involved in inflammation, tissue homoeostasis and cellular repair (Thun *et al.,* 2002). Prostaglandins also play roles as co-carcinogens and tumour promoters (Thun *et al.,* 2002). There are two forms of the COX enzymes: cyclo-oxygenase 1 (COX-1) and cyclo-oxygenase 2 (COX-2). COX-2 and its main metabolite prostaglandin E_2 (PGE_2) have been found to be up-regulated in various tumours and COX-2 knockout mice models result in inhibition of cancer suggesting this enzyme plays a key role in tumour development. In addition to this, COX-2 induction is associated with invasion and angiogensis and PGE_2 enhances tumourigenesis. COX-2 and PGE_2 expression correlates with increased angiogenesis and invasion. COX-2 is induced by mediators of inflammation, mitogens and cytokines which result in inflammation, pain and fever. NSAIDs are thought to be involved in inhibiting the production of the prostaglandins by inhibiting the COX-2 enzyme and therefore suppressing the tumour-promoting activity of prostaglandins, reversing prostaglandin-induced immunosuppression and preventing the activation of carcinogens by the peroxidase component of COX enzymes (Young, 1994; Schiff and Regas, 1997). Interestingly, regular aspirin intake has been shown to reduce the risk of colorectal cancer that overexpress COX-2 but appears to have no effect on the risk of colorectal cancers with weak or absent expression of COX-2 (Chan *et al.,* 2007).

However, the chemoprotective effects of aspirin cannot be solely attributed to its ability to inhibit COX since its anti-proliferative effects have been observed in cells without COX activity. In addition, several NSAID derivatives which do not inhibit COX still have anti-proliferative effects

(Gardner *et al.,* 2004). This suggests that aspirin may be affecting other cellular pathways and there is increasing evidence to support this.

Figure 1.4 Arachidonic acid metabolism. NSAIDs including aspirin have been shown to block metabolism of arachidonic acid by inhibiting COX enzymes and therefore preventing tumourgenesis. (PGD_2 = prostaglandin D_2; PGE_2 = prostaglandin E_2; $PGF_{2\alpha}$ = prostaglandin $F_{2\alpha}$; TXA_2 = thromboxane A_2; PGI_2 = prostaglandin I_2). (Figure adapted from Thun *et al.,* 2002).



1.3.2.2 Nuclear Factor kappa B (NFκB) signalling pathway

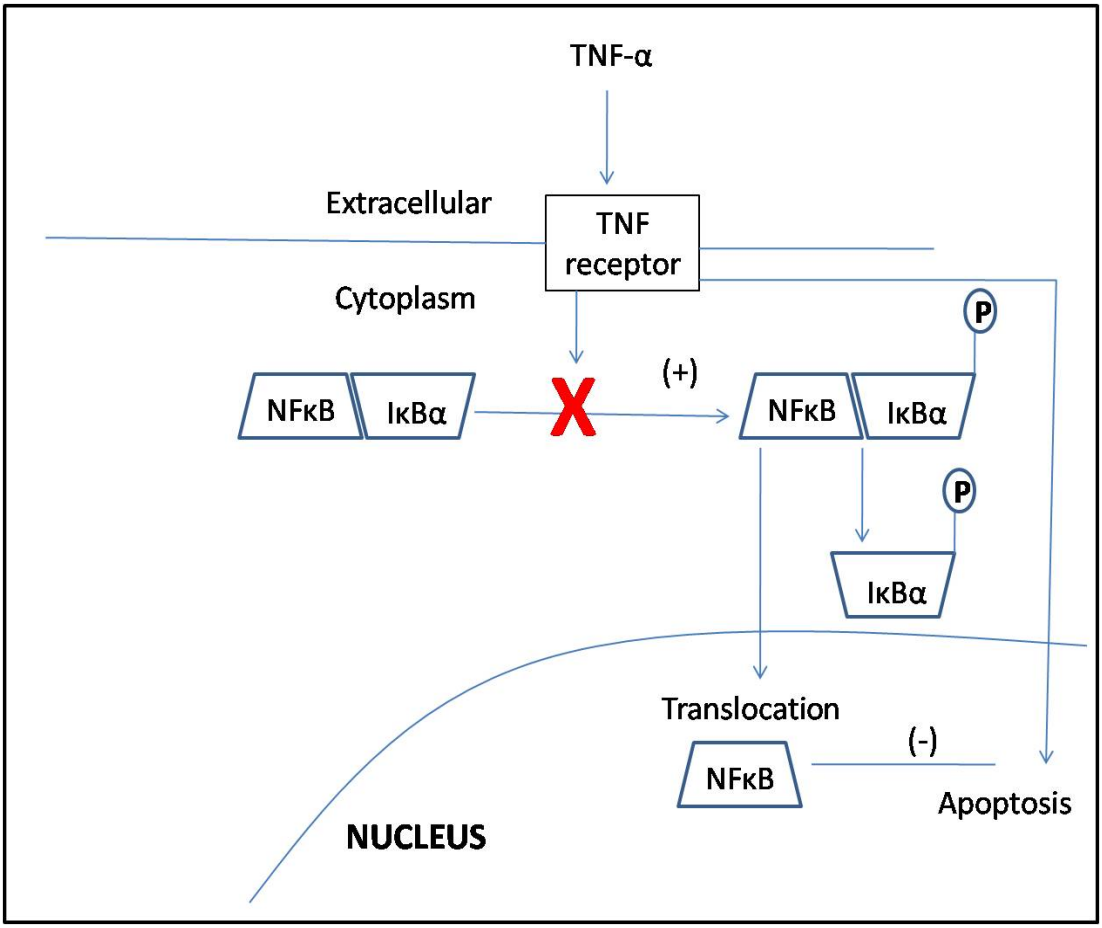
Aspirin has been shown to alter the Nuclear Factor kappa B (NFκB) signalling pathway in colon cancer cells (Stark *et al.,* 2001; Stark *et al.,* 2007, Din *et al.,* 2004; Kopp and Ghosh, 1994) independent of COX expression. NFκB is a transcription factor that activates genes involved in the regulation of inflammatory responses and apoptosis and has been found to be involved in the development of colorectal cancer (Greten *et al.,* 2004). NFκB promotes cell growth by activating expression of cyclin D1 which regulates the G1 to S phase progression of the cell cycle and regulates the cell adhesion molecule ICAM-1 and therefore it is involved in the metastatic processes of colorectal cancer. Essentially, NFκB is a cytoplasmic protein complex of Re1A and p50 which is held in the cytoplasm by the inhibitor protein IκB. When triggered by proinflammatory cytokines such as TNF-α, IκB is phosphorylated by IκB kinase (IKK) which leads to the subsequent degradation of NFκB. This leads to the translocation of the dissociated NFκB from the cytoplasm to the nucleus where it activates the expression of specific cellular genes (Figure 1.5).

Stark *et al.,* (2001) have shown that aspirin activates the NFκB pathway. The study shows that aspirin reduced cytoplasmic IκBα levels in a dose dependant manner correlating with increased cell death. In addition, the study demonstrated that along with decreased levels of IκBα there was an increase in NFκB suggesting that aspirin activates the NFκB pathway. They suggest that aspirin induces NFκB nuclear translocation which is responsible for subsequent apoptosis. They also show that IκBα degradation by aspirin is cell type specific and this may account for differences seen in aspirin response to cell death in different cell types by epidemiological studies and could account for the specific sensitivity of colon cancer cell to aspirin. This is further supported by subsequent studies which suggest that IκBα degradation and nuclear translocation is specific to colorectal cancer cells irrespective of COX-2 levels, p53 status and MMR status (Din *et al.,* 2004; Din *et al.,* 2005). In addition, Stark *et al.,* (2006) have shown in animal models aspirin induced degradation of IκBα in a time dependent manner and hence the activation of the NFκB pathway.

In contrast to these findings, NFκB has also been shown to be inhibited upon aspirin treatment in colorectal cancer cells and therefore inhibit the subsequent inflammatory responses (Kopp and Ghosh., 1994; Yin *et al.,* 1998; Yamamoto *et al.,* 1999). These studies show that the NFκB pathway is inhibited by aspirin as it specifically binds to IKKβ and therefore inhibits its kinase

activity. This prevents IκB degradation and therefore subsequent nuclear translocation of NFκB (Yin *et al.,* 1998; Yamamoto *et al.,* 1999). The reason for this conflict in findings may be that Stark *et al.,* (2001) analysed the effects of NFκB signalling specifically whereas other studies have analysed the effects on cytokine mediation of NFκB in response to aspirin.

Figure 1.5. Brief schematic overview of NFκB pathway (Figure adapted from McDade *et al.,* 1999).



1.3.2.3 Bcl-2/Bax proteins and apoptosis

There is also evidence that aspirin down-regulates Bcl-2 expression and subsequently promotes tumour necrosis factor-related apoptosis-inducing ligand-induced (TRAIL) apoptosis (Kim *et al.,* 2005). Bcl-2 is an anti-apoptotic molecule which inhibits the release of cytochrome *c* from mitochondria into the cytoplasm. Bcl-2 expression is regulated by NFκB and as aspirin was found to down-regulate *Bcl-2* gene expression it was therefore hypothesised that this decrease in Bcl-2 expression was due to NFκB inhibition (Kim *et al.,* 2005). Indeed the study found that aspirin inhibited IKKβ activity, dephosphorylated IkBα, increased the level of IκBα and prevented NFκB nuclear translocation (Kim *et al.,* 2005). In addition, this study showed that the apoptotic effects of the cytotoxic molecule TRAIL were enhanced by pre-treatment with aspirin. It is thought that this may be due to the down-regulation of *Bcl-2* gene expression, which leads to the subsequent activation of caspases, translocation of Bax and the release of cytochrome *c* (Kim *et al.,* 2005). Indeed, the induction of translocation of Bax to mitochondria upon aspirin treatment has been previously suggested (Zimmermann *et al.,* 2000). Lai *et al.,* (2008) have also shown that aspirin may inhibit proliferation and promote apoptosis of COX-2 negative colon cancer cells by up-regulating Bax expression and down-regulating Bcl-2.

Cytochrome *c* release and loss of mitochondrial membrane potential has also been suggested by other studies which propose that aspirin induces cytochrome *c* release and the translocation of the Bax protein from the cytosol into the mitochondrial matrix (Pique *et al.,* 2000 and Redlak *et al.,* 2005). Redlak *et al.,* (2005) showed that NSAIDs can disrupt oxidative phosphorylation in the respiratory chain and cause mitochondrial disintegration such as depolarization and outer membrane permeability, which gives rise to the release of cytochrome *c* from the mitochondria to the cytosol, and induces translocation of Bax from the cytosol into the mitochondria. In addition, it has also been shown that aspirin may inhibit proteasomal function which induces the mitochondrial abnormalities by causing changes in mitochondrial membrane potential, release of cytochrome *c*, activation of caspase-9 and caspase-3 and leads to the subsequent activation of apoptosis (Dikshit *et al.,* 2006).

1.3.2.4 Inhibition of cytosolic phospholipase A_2 expression

Phospholipase A_2 is a family of enzymes which generates free fatty acids such as arachidonic acid and therefore plays a role in the COX pathway. Yu *et al.,* (2003) showed that aspirin treatment of the COX-2 negative colorectal cancer cell line SW480, decreased PGE_2 levels and also cytosolic phospholipase A_2 ($cPLA_2$) mRNA levels in a concentration dependent manner. This study highlights that aspirin is able to have anti-proliferative and apoptotic effects on cells regardless of COX expression and therefore suggest that aspirin has a COX independent mechanism of action (Yu *et al.,* 2003).

1.3.2.5 Depletion of intracellular polyamines

Hughes *et al.,* (2003) showed that NSAIDs treatment of colorectal cancer cell lines was not only cytotoxic but also caused a decrease in intracellular polyamine content. Polyamines are growth factors and involved in protein synthesis. An increase in intracellular polyamine concentration is observed in the early stages of carcinogenesis. Interestingly, when polyamines were re-introduced to the treated cells, apoptosis was inhibited suggesting that the polyamine pathway is affected by NSAID treatment in colorectal cancer cells and that modulation of this pathway may explain the chemoprotective effects of NSAIDs (Hughes *et al.,* 2003).

1.3.2.6 Antioxidant activity of aspirin

One other mechanism suggested is the intrinsic antioxidant activity of aspirin preventing double stranded DNA breaks. Utilising a model system to induce oxidative DNA damage, Hsu and Li (2002) showed that addition of aspirin inhibited the DNA strand breaks in a concentration dependent manner. This suggests that aspirin protects against oxidative DNA damage and this may contribute to its anti-cancer effects (Hsu and Li, 2002).

1.3.2.7 Increase in Rac1 expression

Hardwick *et al.,* (2004) analysed changes to gene expression utilising DNA microarray in the colorectal cancer cell line HT-29 upon aspirin treatment and found a significant increase in *Rac1* gene and also protein expression in a time and concentration dependant manner. Rac1 is involved in intestinal epithelial cell differentiation (Stappenbeck and Gordon, 2000) and high

expression of *Rac1* is found in mature colon cells undergoing apoptosis as observed in mouse and human colon (Hardwick *et al.,* 2004). The significance of this increase suggests that aspirin may promote cell differentiation by increasing *Rac1* expression (Hardwick *et al.,* 2004).

1.3.2.8 Increased NSAID-activated gene (NAG-1) protein

NSAID-activated gene (NAG-1) protein a member of the transforming growth factor beta (TGF-β) family, also known as macrophage inhibitory cytokine-1 (MIC-1), placental transforming growth factor β (PTGF-β) and prostate-derived factor (PDF) (Bootcov *et al.,* 1997; Lawton *et al.,* 1997; Paralkar *et al.,* 1998) has been reported to be up-regulated by NSAIDs such as aspirin (Tesei *et al.,* 2008; Baek *et al.,* 2001a; Baek *et al.,* 2001b; Kim *et al.,* 2002). This up-regulation of NAG-1 induces apoptosis and also suppresses the growth of xenografts in nude mice (Baek *et al.,* 2001b).

1.3.2.9 Induction of G1 arrest via activation of Ataxia-telangiectasia-mutated kinase (ATM)

Aspirin may induce a G1 arrest and apoptosis by activating p53 and p21 in an ATM dependent manner. The significance of this is that by activating these checkpoint pathways, aspirin may retain uncontrolled proliferation of colorectal cancer cells, so enhancing their response to stresses such as DNA damage and promoting entry of abnormal cells into apoptosis (Luciani *et al.,* 2007)

1.3.2.10 Selection for microsatellite stability

A key suggestion for the anti-tumour mechanism of action of aspirin may be via interaction with the DNA repair systems. This is of key interest particularly in the context of colorectal cancer where DNA repair defects are a known cause. It has been shown that the MSI mutator phenotype is suppressed by aspirin. Aspirin treatment of the colorectal cancer cell line HCT116 which is MMR deficient with defects in MLH1, MSH2 and MSH6 reduced the MSI phenotype and there was induction of apoptosis (Ruschoff *et al*., 1998). It is speculated that aspirin exposure resulted in genetic selection of cells with MSI and that MSI unstable cells were selected out by apoptosis (Ruschoff *et al.,* 1998). This observation is important as microsatellite instability resulting from a genetic mutation in MLH1, accounts for 5 - 10% of colonic tumours.

1.3.2.11 Induction of the DNA mismatch repair (MMR) proteins

In addition to the study on aspirin affects on MSI (Ruschoff *et al.,* 1998), it has also been observed that aspirin affects the DNA mismatch repair proteins MLH1, MSH2, MSH6 and PMS2 in DNA MMR proficient cells, which ultimately facilitates programmed cell death (Goel *et al.,* 2003). Aspirin treatment inhibited the growth of cancer cells and additionally showed that the expression of mismatch repair protein, MLH1 and also PMS2 increased in the mismatch repair protein proficient cell lines studied. This suggests that aspirin may directly or indirectly interact with these mismatch repair proteins to regulate cell survival (Goel *et al.,* 2003). This is a highly significant observation as defects in MMR proteins are ultimately responsible for HNPCC and this study speculates in particular on the involvement of MLH1 regulation in the chemoprotective effects of aspirin. In addition to this, recent evidence has shown that although MLH1 expression is decreased in cases of sporadic colorectal adenoma, MLH1 expression was found to be increased in cases of sporadic colorectal adenomas with regular aspirin use (Sidelnikov *et al.,* 2009).

Identifying and understanding pathway modulation by aspirin is key to understanding its chemopreventative effect on colorectal cancer. The suggestion that aspirin affects DNA repair proteins is therefore of central interest and has so far received relatively little attention. It is therefore logical to investigate the potential role of genes known to be mutated in HNPCC like *MLH1.*

Table 1.2 Summary of potential mechanisms for aspirin inhibition of colorectal cancer.

Mechanism	References
Cyclo-oxygenase inhibition	Smith *et al.,* 2000; Ferrandez *et al.,* 2003; Chan *et al.,* 2007
Nuclear Factor Kappa B (NFkB) signalling pathway	Stark *et al.,* 2001; Stark *et al.,* 2007; Din *et al.,* 2004; Kopp and Ghosh, 1994; Yamamoto *et al.,* 1999
Bcl-2/Bax proteins and apoptosis	Kim *et al.,* 2005; Lai *et al.,* 2008; Pique *et al.,* 2000; Redlak *et al.,* 2005; Dikshit *et al.,* 2006
Inhibition of cytosolic phospholipase A_2 expression	Yu *et al.,* 2003
Depletion of intracellular polyamines	Hughes *et al.,* 2003
Antioxidant activity of aspirin	Hsu and Li, 2002
Increase in Rac1 expression	Hardwick *et al.,* 2004
Increased NSAID-activated gene (NAG-1) protein	Tesei *et al.,* 2008; Kim *et al.,* 2002; Baek *et al.,* 2001
Induction of G1 arrest via activation of Ataxia-telangiectasia-mutated kinase (ATM)	Luciani *et al.,* 2007
Selection for microsatellite stability	Ruschoff *et al.,* 1998
Induction of the DNA mismatch repair (MMR) proteins	Goel *et al.,* 2003

1.4 MutL homologue 1, MLH1

A germ - line mutation in *MLH1* is common in patients with HNPCC and is also found in many sporadic colorectal tumours (Kuismanen *et al.,* 2000; Jacob and Praz, 2002). Approximately 200 mutations of MLH1 have been described in patients with HNPCC and this represents 55% of mutations which have been identified (Jacob and Praz, 2002).

The *MLH1* gene situated on chromosome 3p21.3 encodes an 84.6 kDa protein. The N-terminus has ATPase activity and the C-terminus acts as the interacting domain with the other MMR proteins such as PMS1, PMS2 and MLH3 (Robinson and Brown, 2003). Many MLH1 mutations found in HNPCC patients occur in this C-terminus region which results in a lack of MLH1 and PMS2 interaction and therefore deficient MMR (Robinson and Brown, 2003). A loss of MLH1 has also been shown to cause resistance to anticancer drugs such as temozolomide and cisplatin (Robinson and Brown, 2003; Darkes *et al.,* 2002) highlighting how dysregulation of MMR proteins can also have a subsequent effect on cancer treatment considerations. Interestingly, MLH1 and also MSH2 over-expression has been shown to induce apoptosis and repair (Zhang *et al.,* 1999). Indeed, MLH1 has been shown to interact with downstream signalling proteins involved in apoptosis initiation (Jiricny and Marra, 2003; MacPartlin *et al.,* 2003). Evidence that MLH1 may be involved in apoptosis regulation is also provided by its interaction with caspase 3. Caspase 3 is a cysteine protease and MLH1 cleavage by capsase 3 causes partial translocation of MLH1 from the nucleus to the cytoplasm resulting in a carboxyl-terminal proapoptotic product suggesting that MLH1 may be involved in DNA damage-induced apoptosis (Chen *et al.,* 2004). However, the breast cancer cell line MCF7 is caspase 3 deficient and therefore MLH1 proteolysis does not occur suggesting apoptosis is induced via another mechanism in this cell line (Chen *et al.,* 2004).

In addition to interacting with proteins in the MMR system (MSH2, PMS1, PMS2, MLH3), MLH1 has also been shown to interact with other MMR associated proteins such as PCNA, Exo1, the Bloom's Syndrome Protein (BLM) and the BRCA1 Associated Genome Surveillance Complex (BASC) (Robinson and Brown, 2003). BLM is mutated in the genetic syndrome Bloom's Syndrome which is characterised by genomic instability and cancer predisposition. Both MLH1 and BLM reside in the BASC complex which also includes MSH2, MSH6, ATM, RFC, BRCA1 and also the RAD50-MRE11-NBS1 complex (Robinson and Brown., 2003). MLH1 and MSH2 have also been shown to interact with c-MYC and MYC associated factor X (MAX) providing evidence that MLH1 interacts with other genomic surveillance systems (MacPartlin *et al.,* 2003). c-MYC is a proto-oncogene

that plays a role in the regulation of cell cycle, differentiation and apoptosis which is regulated by its interaction with MAX. The interaction of MLH1 with these proteins suggests that MLH1 may cross-talk with proteins involved in apoptotic mechanisms which may affect MMR. Further evidence that MLH1 may play a role in apoptosis is provided by the interaction of MLH1 with ATM, a key protein involved in apoptosis. Over-expression of MLH1 has been shown to induce apoptosis possibly due to its interaction with ATM (Luo *et al.,* 2004; Zhang *et al.,* 1999).

It is therefore clear that although it is known that MLH1 is required for MMR, a definition of its precise role especially concerning apoptotic mechanisms, has yet to be fully elucidated and so identifying interacting partners may help to ascertain this. One interesting MLH1 interaction observed is with the methyl-CpG binding domain protein 4 (MBD4) (Bellacosa *et al.,* 1999).

1.5 Methyl-CpG Binding Domain Protein 4 (MBD4)

MBD4 is a 66 kDa mammalian methyl-CpG binding protein (also referred to as Methyl-CpG Binding Endonuclease I or MED1) and is a member of the methyl-CpG-Binding Domain (MBD) protein family which includes methyl-CpG-binding protein 2 (MECP2), methyl-CpG-binding domain protein 1 (MBD1), methyl-CpG binding domain protein 2 (MBD2) and methyl-CpG-binding domain protein 3 (MBD3). It was identified in a database search aimed at annotating proteins with an MBD region known to have selective affinity for methylated DNA (Hendrich and Bird, 1998). Cytosine-phosphate-Guanine (CpG) islands are regions of DNA that are rich in Guanine (G) and Cytosine (C) dinucleotides. CpG methylation is the most common epigenetic modification which can occur in vertebrate genomes (Ballestar and Wolffe, 2001). The MBD is able to bind to single methylated CpG pairs and the presence of an MBD indicated possible involvement of MBD4 in DNA repair associated with methylated CpG sites. Potentially mutagenic G:T mismatches can arise due to deamination of the methylation product, 5-methylcytosine (m^5C) to thymine (Scharer and Jiricny, 2001) and it is suggested that MBD4 is involved in repairing m^5C deamination at methylated CpG sites (Hendrich *et al.,* 1999). MBD4-deficient mice demonstrated an increase in C-T transitions at CpG sites and therefore MBD4 acts to suppress CpG mutability (Millar *et al.,* 2002; Wong *et al.,* 2002) and in essence acts as a guardian of CpG regions.

As well as an amino-terminal MBD domain, MBD4 also has a carboxy-terminal region that shows homology to the glycosylase/endonuclease domains of bacterial repair proteins (Bellacosa *et al.,* 1999). Two possible functions of MBD4 were originally considered, both DNA glycosylase and endonuclease activity. It was originally proposed that MBD4 may be a putative homologue of MutH and necessary for strand discrimination as it is able to recognise hemimethylated DNA. However, experimental evidence demonstrated that MBD4 lacked endonuclease activity and therefore does not play a role in MMR strand discrimination (Drummond and Bellacosa, 2001).

1.5.1 Role of MBD4 in Base Excision Repair

MBD4 does demonstrate the ability to excise Thymine (T) and Uracil (U) from guanine-thymine (G:T) and guanine-uracil (G:U) mismatches in a CpG context indicating MBD4 is a specific T/U DNA glycosylase (Hendrich *et al.,* 1999). In addition, MBD4 has also shown glycosylase activity for 5-fluorouracil (5-FU), 3,N^4-ethenocytosine and 5-chlorouracil (ClU) when mismatched with guanine and also halogenated bases (Petronzelli *et al.,* 2000a; Petronzelli *et al.,* 2000b; Hendrich *et al.,*

1999; Meyers *et al.,* 2005; Turner *et al.,* 2006; Valinluck *et al.,* 2005). Also, MBD4 has been shown to repair Thymine glycol (Tg) and Guanine mismatches produced by oxidation of m^5C (Yoon *et al.,* 2003). Therefore since it was identified as an MLH1 binding protein, MBD4 is now considered to be a DNA glycosylase involved in base excision repair (BER), specifically within a CpG context. BER is a system specifically responsible for excising damaged or mismatched bases, using DNA glycosylases, and the subsequent repair. DNA glycosylases catalyse the hydrolysis of the N-glycosyolic bonds linking the altered or damaged base to the deoxyribose-phosphate backbone. This results in the excision of the free base and generates a site of base loss called apurinic or apyrimidinic (AP) sites. The removal of AP sites is initiated by another class of enzymes called apurinic/apyrimidinic (AP) endonucleases (APE) which specifically recognise these sites. This APE incision leaves a nucleotide gap which is filled by DNA polymerase and then sealed by DNA ligase.

1.5.2 The domains of MBD4

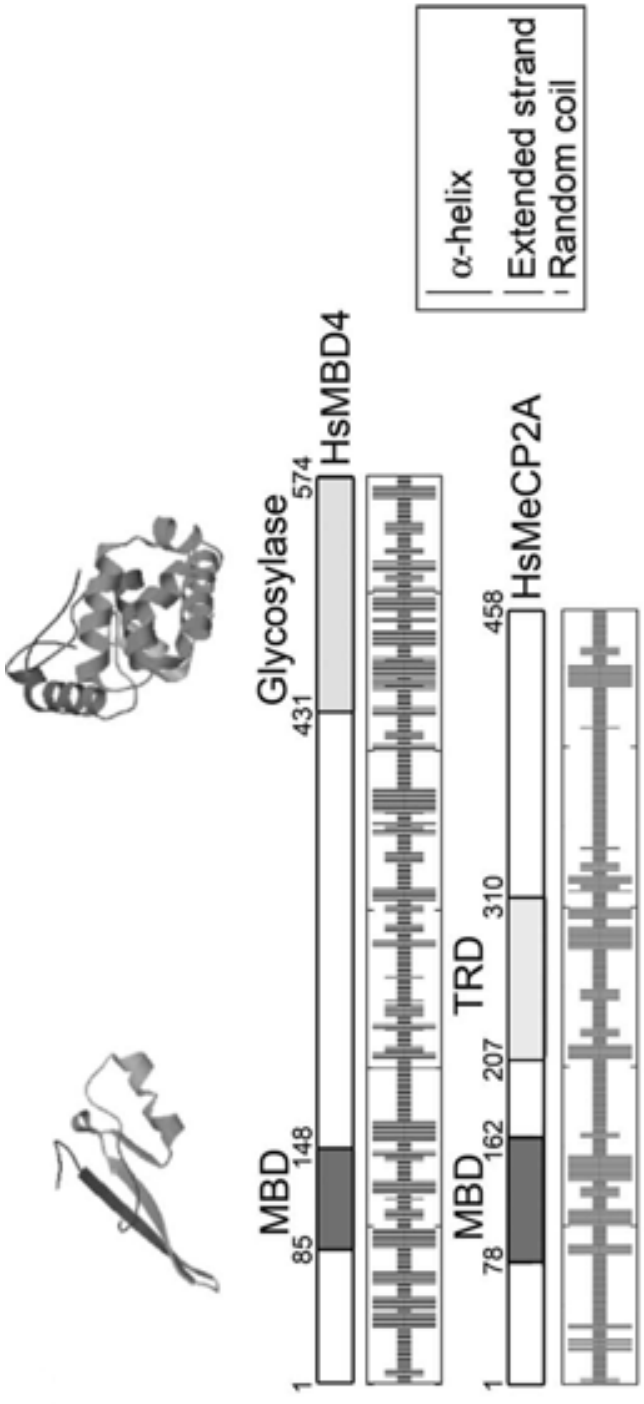
It has been shown that the two domains of MBD4 can act independently of each other (Kondo *et al.,* 2005; Petronzelli *et al.,* 2000a). A comparison of the structure of MBD4 compared with the structure of MeCP2 shows that although both proteins share a common MBD, MBD4 has a very different structure due to its additional glycosylase domain (figure 1.6). In addition the structure of MBD4 is more organised compared to that of MeCP2 (Ishibashi *et al.,* 2008). This would suggest that although they may share a common functional feature, the glycosylase domain of MBD4 and also the structural differences between MBD4 and MeCP2 may mean that MBD4 has different binding abilities to DNA (Ishibashi *et al.,* 2008). Ishibashi *et al.,* (2008) tested this hypothesis and have shown that MBD4 does indeed differ to MeCP2 in the way it binds to DNA. MeCP2 binds specifically to nucleosomes containing methylated DNA whereas MBD4 bound non-specifically. In addition, although MBD4 has been shown to bind to methylase recognition sites, there is conflicting evidence about whether MBD4 activity is affected by the presence of a methyl group opposite a mismatch Thymine (Hendrich *et al.,* 1999; Petronzelli *et al.,* 2000a; Turner *et al.,* 2006; Valinluck *et al.,* 2005).

MBD4 preferentially binds to T:G mismatches where the CpG region is hemimethylated (Turner *et al.,* 2006) suggesting that the MBD domain and the glycosylase domain may work synergistically with the MBD allowing MBD4 to specifically target regions of heavy cytosine methylation where mismatches are most likely to take place (Ishibashi *et al.,* 2008). However, studies have shown that this may not be the case (Drummond and Bellacosa, 2001). The structure of MBD4 would

suggest that the MBD and the glycosylase domain are unlikely to simultaneously bind to the same dinucleotide and this led to the suggestion that the MBD allows MBD4 to target methylated DNA regions of the genome so that it is present in the event of a T:G mismatch (Petronzelli *et al.,* 2000a; Wu *et al.,* 2003). However, Ishibashi *et al.,* (2008) have shown that the methylation status has a minimal effect on the glycosylase activity of MBD4. They also demonstrated that the presence of a nucleosome decreased the glycosylase efficiency of MBD4 (Ishibashi *et al.,* 2008).

It has also been suggested that MBD4 may play a role in transcriptional repression (Kondo *et al.,* 2005; Kondo *et al.,* 2006). MBD4 has been shown to specifically bind to highly methylated promoters of the *MLH1* and also *CDKN2A* genes. This suggests that MBD4 may play a role in transcriptional silencing in cancer.

Figure 1.6 Structural comparison of MBD4 and MeCP2. Although both MBD proteins, MBD4 and MeCP2 have different structures. The box drawings are a schematic representation of the functional domain organization of human MeCP2 and MBD4. The secondary structures predicted from the amino acid sequences by using the hierarchical neural network are shown underneath. The tertiary-structure organizations of the methyl-CpG-binding and glycosylase domains of human MBD4 as predicted from the crystallographic data on the methyl-CpG-binding domain (MBD) of human MBD1 and the mouse glycosylase domain of MBD4 are shown above the corresponding schematic linear representations. Tertiary-structure predictions were carried out using the SWISS-MODEL server. (TRD = transcription repression domain; Hs, *Homo sapiens*) (Figure taken from Ishibashi *et al.,* 2008).



1.5.3 Interaction of MBD4 with MLH1 and potential role in apoptosis

Unlike other DNA glycosylases, MBD4 is reported to be able to bind to the MMR protein MLH1 via its glycosylase domain (Bellacosa *et al.,* 1999; Kondo *et al.,* 2005). Alongside its discovery as an MBD protein, MBD4 was also independently identified based on its interaction with the MMR protein MLH1 (Bellacosa *et al.,* 1999). A yeast two-hybrid system to determine novel interacting proteins with MLH1 identified an interaction with the MED1 gene, encoding a 580 amino acid protein containing two regions and was recognised to be MBD4 (Bellacosa *et al.,* 1999). This interaction has subsequently been supported by the work of MacPartlin *et al.,* (2003) and suggests a possible link between MBD4 and MMR. MBD4 may play a role in coordinating BER and MMR, for example when G:T mismatches occur during DNA replication (Bellacosa, 2001). MLH1 is known to induce apoptosis triggered by DNA damage (Hardman *et al*., 2001). Considering the interaction of MBD4 with MLH1, it could be suggested that MBD4 may also have a role like MLH1, in regulating apoptosis and genomic surveillance. This speculation is further supported by the interaction of MBD4 with Fas-associated death domain protein (FADD) (Screaton *et al.,* 2003). The interaction of MBD4 with FADD suggests that MBD4 may play a role in regulating apoptosis although a mechanism has yet to be elucidated. Indeed, MBD4 deficiency has been shown to reduce the apoptotic response to DNA-damaging agents in the murine small intestine (Sansom *et al.,* 2003). *MBD4* expression has also been analysed in the T cells of patients with systemic lupus erythematosus and was found to be increased (Balada *et al.,* 2007). It is speculated that due to its association with FADD and therefore the apoptotic pathway, this increase of MBD4 may account for the abnormal increase in cell death rate observed in patients with systemic lupus erythematosus. Interestingly, the study also found that *MBD4* gene transcription inversely correlated with age in the control population suggesting that MBD4 expression decreases during adulthood and this may account for the increase in mutations at CpG regions during adulthood and subsequent cancer risk (Balada *et al.,* 2007). Deficiency in MBD4 in mouse embryonic fibroblasts caused expression of proteins involved in MMR (MSH2, MSH6, MLH1 and PMS2) to be down-regulated suggesting that MBD4 deficiency may account for the lack of apoptotic response in those cells to DNA damaging agents. Interestingly, down-regulation of the MMR proteins was not noted at an mRNA level, however this study does provide evidence that MBD4 may be a link between BER and MMR (Cortellino *et al.,* 2003).

To further support the role of MBD4 along with its interaction with MLH1 in apoptosis, a recent study has shown that over-expression of MBD4 mRNA in early *Xenopus* embryos induced an

apoptotic response that disrupted early development, as did an over-expression of MLH1 mRNA (Ruzov *et al.,* 2009). Ruzov *et al.,* (2009) also demonstrated that MBD4 and MLH1 co-localize at sites of DNA damage and that the apoptotic response was signalled via MBD4 and its interaction with MLH1 suggesting that MBD4 acts as an apoptotic-signalling molecule. In addition they also demonstrated that MBD4 and MLH1 colocalise with the DNA methyltransferase DNMT1 which is involved in MMR and DNA damage response pathways (Guo *et al.,* 2004; Palii *et al.,* 2008). MBD4 expression has previously been linked with DNA methyltransferase (DNMT) expression in male and female fetal gonads, supporting a role for MBD4 in chromatin remodelling of methylated genome sequence (Galetzka *et al.,* 2006). The interaction of MBD4 and MLH1 with DNMT1 suggests that MBD4 and MLH1 may be involved in a DNA damage sensing pathway. In particular the localisation of these proteins at sites of DNA damage suggests that they may play a role in mediating the decision as to whether lesions are repaired or the cell should go into apoptosis. MBD4 has been shown not to bind to methylated MLH1 promoters in endometrial cancer suggesting it is not involved in methylated MLH1 promoter inactivation (Xiong *et al.,* 2006).

1.5.4 Role of MBD4 in MMR and cancer pathogenesis

Further evidence of a possible role of MBD4 in the MMR system and also its potential involvement in the pathogenesis of colorectal cancer was provided by research which demonstrated that between 20 and 43% of primary human colorectal carcinomas that showed MSI also had inactivating mutations in MBD4 (Riccio *et al.,* 1999; Bader *et al.,* 1999; Yamada *et al.,* 2001). Many of these tumours were HNPCC tumours which also carried MLH1 mutations or sporadic tumours which also had MLH1 and MSH2 mutations. In addition, it was also demonstrated that MBD4 mutations frequently occurred in other cancers which demonstrated MSI such as gastric, endometrial and pancreatic carcinomas (Riccio *et al.,* 1999). Many of the mutations of MBD4 found in these tumours have resulted in a truncated protein product which lacked the C-terminal catalytic domain (Riccio *et al.,* 1999). These studies would suggest that MBD4 may act as a tumour suppressor. The role of MBD4 in DNA repair *in vivo* was assessed by generating a mouse line with an inactivating mutation in MBD4. However, MBD4 inactivation did not cause MSI in the murine genome suggesting that inactivating mutations in MBD4 alone do not impact on the initiation of tumourgenesis (Wong *et al.,* 2002) although lack of *MBD4* did increase tumorigenicity (Wong *et al.,* 2002; Millar *et al.,* 2002).

Although MBD4 mutations have been found in MMR-deficient tumours with MSI (Bader *et al.,* 2000; Bader *et al.,* 1999; Evertson *et al.,* 2003), it has also been demonstrated that MBD4 mutations do not affect tumourgenesis of MMR-deficient tumours (Sansom *et al.,* 2004). Over-expression of MBD4 has been associated with MSI (Drummond and Bellacosa, 2001; Screaton *et al.,* 2003) and over-expression has also been shown to be positively correlated with glioma malignancy (Schlegel *et al.,* 2002). Interestingly, a down-regulation of MBD4 correlates with poor tumour differentiation in hepatocellular carcinoma (Saito *et al.,* 2001). *MBD4* gene expression has been found to be decreased in sporadic colorectal cancers thought to be due to promoter methylation suppressing MBD4 expression (Howard *et al.,* 2009). One study has highlighted that changes observed in gene expression of *MBD4* in small cell lung cancers and non-small cell lung cancers were dependent on the normalisation of the data to specific reference genes (Sato *et al.,* 2002). The results for individual genes could alter dramatically from up-regulated to down-regulated, dependent on the reference gene chosen to normalise the data, so suggesting caution should be taken when analysing results from studies of this nature (Sato *et al.,* 2002). A further study also showed that MBD4 mutations were unlikely to be involved in the pathogenesis of hyperplastic polyposis syndrome (HPS), a syndrome characterised by multiple large and/or proximal hyperplastic polyps, although single nucleotide polymorphisms of MBD4 were identified (Chow *et al.,* 2006). Further to this, MBD4 polymorphisms have been shown to be associated with lung cancer risk and esophageal cancer risk (Shin *et al.,* 2006; Hao *et al.,* 2004) although there are contrasting findings suggested to be due to the difference in ethnic groups that were analysed (Shin *et al.,* 2006).

Cortellino *et al.,* (2003) also provide evidence that MBD4 may regulate cell cycle response to DNA damage. They showed MBD4 deficient cells undergo apoptosis in response to DNA damaging agents including chemotherapeutic agents and as such demonstrated that MBD4 deficiency may need to be considered in therapeutic approaches when treating cancer. A potential role of MBD4 as a prognostic marker for predicting colorectal cancer patient survival undergoing 5-FU treatment has been suggested (Ju *et al.,* 2006). Interestingly, a microarray study showed that *MBD4* gene expression was up-regulated in small intestine polyps from ApcMin/+ mice upon treatment with the isothiocyanate sulforaphane. ApcMin/+ mice develop spontaneous pre-neoplastic intestinal polyps due to a mutation in the *APC* gene commonly found in colorectal cancer. Epidemiological studies have shown that isothiocyanates have been linked with cancer chemoprevention and sulforhane specifically has been shown to prevent the growth of polyps in ApcMin/+ mice. (Khor *et al.,* 2006). The up-regulation of *MBD4* therefore suggests that its

expression may be regulated by isothiocyanates and may play a role in the subsequent chemopreventative effect of colorectal cancer.

A premature truncation of MBD4 due to a naturally occurring frameshift of a polynucleotide tract which results in a premature stop in translation has been recognised in human cells and is thought to act in a dominant negative manner, competitively inhibiting glycosylase activity of wild type MBD4. Over-expression of this truncated form in cells increased mutation frequency (Bader *et al.,* 2007), led cells to acquire structural chromosomal rearrangements and increased resistance of the cells to the drug etoposide (Abdel-Rahman *et al.,* 2008). Etoposide inhibits topoisomerase II activity which leads to a subsequent increase in double strand breaks and chromosomal translocations and so this study suggests that MBD4 may play a role in maintaining chromosomal stability (Abdel-Rahman *et al.,* 2008).

1.5.5 Identification of sMBD4

Recently, a novel alternatively spliced form of MBD4 has been identified and termed sMBD4 (Owen *et al.,* 2007). sMBD4 lacks exon 3 of MBD4 and unlike other alternatively spliced forms of MBD4 reported in human and murine transcripts (Hendrich and Bird., 1998) lacks the MBD domain (see Chapter 3, Figure 3.1). The chicken MBD4 also appears to lack the MBD (Zhu *et al.,* 2000). Like MBD4, sMBD4 shows G:U activity but fails to demonstrate G:T activity. However, lack of G:T activity has previously been shown by a genetically engineered MBD4 without an MBD domain (Hendrich *et al.,* 1999). Owen *et al.,* (2007) supported the suggestion that MBD4 may be the evolutionary result of a glycosylase and MBD fusion (Hendrich *et al.,* 1999). However, a specific role for sMBD4 has yet to be elucidated.

1.5.6 Potential involvement of MBD4 in disease pathogenesis

As a recently discovered protein, much is to be learnt about MBD4 and many studies have been carried out in order to analyse its significance particularly in the pathogenesis of disorders other than cancer. MBD4 expression is increased in GABA cells from the hypothalamus of both schizophrenia and bipolar disorder, suggesting that MBD4 may play a role in suppressing key transcriptional complex associated with the cell cycle (Benes *et al.,* 2009). It was postulated that MBD4 may play a role in somatic hypermutation and class switch recombination on account of its glycosylase activity and association with MLH1 (Bardwell *et al.,* 2003). However this study

utilising MBD4 knockout mice showed that MBD4 is not involved antibody diversification (Bardwell *et al.,* 2003).

In summary, the body of research on MBD4 has provided evidence of it being involved in BER, MMR, DNA damage response as well as transcriptional repression. However further research is needed to extend the knowledge about this protein and its functional role. Of particular interest is the interaction of MBD4 with MLH1. The key finding that MLH1 may play a role in the anti-tumourgeneic effects of aspirin (Goel *et al.,* 2003) leads to the question as to whether MBD4 is also involved.

1.6 DNA Repair Signalling Pathways

The involvement of the MMR system in the pathogenesis of colorectal cancer is now well established and therefore the hypothesis that aspirin may potentially modulate this pathway to prevent carcinogenesis (Goel *et al.,* 2003) is of interest and merits further investigation. As is evident, many MMR proteins interact with proteins from other DNA repair and damage signalling pathways, for example MBD4 and MLH1. As proteins of the MMR system have been found to be induced upon aspirin treatment, it is therefore of interest whether aspirin effects proteins of other DNA repair pathways. Previously, Hardwick *et al.,* (2004) have reported that many cell cycle related genes and also *NFκB* are repressed upon aspirin treatment and Iizaka *et al.,* (2002) have reported an increase in *GADD45α* upon treatment with the NSAID sulindac. However, although, microarray analysis has been previously carried out to determine the effects of aspirin on colorectal cancer (Hardwick *et al.,* 2004; Iizaka *et al.,* 2002), these studies have not looked specifically at DNA damage signalling pathways. The availability of PCR arrays focused on specific pathways now allows for analysis of multiple gene expression within related pathways utilising PCR array technology. The Human DNA Damage Signalling Pathways RT^2 Profiler^{TM} array (SuperArray Biosciences Corporation, Frederick, MD) allows for the analysis of genes specifically related to DNA damage signalling pathways. The genes included on the array are associated with the ATR/ATM signalling pathway and also targets of DNA damage response. Genes involved in apoptosis, cell cycle regulation, such as cell cycle arrest and cell cycle checkpoint and DNA repair, such as damaged DNA binding, base excision repair, double-strand break repair and mismatch repair are included on the array. Therefore in addition to analysing the effects of aspirin on *MBD4* expression the availability of this RT^2 Profiler^{TM} array gives an opportunity to test a wider range of potential DNA repair genes and signalling pathways.

In addition, although microarray analysis has identified key genes with a potential involvement in the mechanism of action of aspirin (Hardwick *et al.,* 2004; Huang *et al.,* 2006; Iizaka *et al.,* 2002; Germann *et al.,* 2003 and Yin *et al.,* 2006) many of these studies have not shown any evidence of a related change in protein expression which is of key significance when considering disease pathogenesis especially as many studies have now shown that gene transcription and protein abundance do not necessarily correlate (Gygi *et al.,* 1999; Lichtinghagen *et al.,* 2002).

1.7 Aims of this study

The overall objective of this study was to explore the possible relationship between aspirin and salicylic acid treatment and induction and behaviour of DNA repair enzymes in relation to their protective effect on occurrence of colorectal cancer.

The biological significance of the recently identified spliced form of MBD4, sMBD4 is to date unknown (Owen *et al.,* 2007). In addition, although previous studies have shown that MBD4 is transcriptionally coupled to MLH1 and it has been shown that MLH1 and MBD4 physically interact to form a complex which can be immunoprecipated (Bellocosa *et al.,* 1999), to date the significance of this interaction has not been fully elucidated. An aim of this research study was to investigate whether an interaction occurred between sMBD4 and MLH1 to help gain further insight into a potential role for sMBD4 and to further define the association between full length MBD4 and MLH1.

A recent study has shown that aspirin treatment increases expression of the DNA repair proteins, MLH1, MSH2 and PMS2 and that the regulation of these key proteins may in part explain the chemoprotective effect of aspirin on colorectal cancer (Goel *et al.,* 2003). Based on the evidence of transcriptional coupling (Bellacosa *et al.,* 1999), as MLH1 expression increases with aspirin treatment (Goel *et al.,* 2003) it would be reasonable to expect that MBD4 expression might also increase with aspirin treatment. Unpublished data (Dr Iain Nicholl: personal communication) showed preliminary evidence of an increase of *MBD4* gene expression in SW480 cells upon salicylic acid treatment by semi-quantitative PCR analysis. A subsequent aim of this research study was to therefore further analyse *MBD4* gene expression in colorectal cancer cell lines upon aspirin and salicylic acid treatment.

The effects of aspirin on DNA damage signalling pathway genes utilising a commercially available PCR array were carried out in order to establish whether other key genes involved in these pathways were affected by aspirin treatment.

Briefly the aims of this study were:

1) To determine whether MLH1 and sMBD4 were binding partners (Chapter 3). Additionally, to determine whether *MBD4* gene expression was increased in colorectal cancer cell line SW480 upon aspirin and salicylic acid treatment utilising semi-quantitative and quantitative real-time PCR (Chapter 4)

2) To utilise novel PCR array experiments to determine if aspirin treatment causes changes in expression of genes involved in DNA damage signalling pathways in the colorectal cancer cell line SW480 (Chapter 4)

3) To determine if changes in gene expression upon aspirin treatment as determined by PCR array experimentation correlated with changes in protein expression (Chapter 5)

Chapter 2

Materials and Methods

2 Materials and Methods

All reagents were purchased from Sigma-Aldrich Company Ltd., (The Old Brickyard, New Road, Gillingham, Dorset SP8 4XT) unless otherwise stated.

2.1 Cell Culture

All cell lines were purchased from the European Collection of Cell Culture (ECACC, Health Protection Agency, Centre for Emergency Preparedness and Response, Porton Down, Salisbury, Wiltshire, SP4 0JG) except HCT116 and HT29 cell lines which were kindly provided by Dr Weiguang Wang (University of Wolverhampton, UK) and the U373MG cell line which was kindly provided by Dr Sarah Jones (University of Wolverhampton, UK).

Cell culture was performed in a Class II hood using aseptic technique and sterile reagents and equipment. Flasks were monitored for cellular morphology and for confluence using an inverted phase contrast microscope (Olympus CK2-TR; Olympus Optical Co., Ltd., Japan). Different cell lines were cultured independently to avoid cross contamination.

2.1.1 Cell Line Resuscitation

Cells were recovered from liquid nitrogen storage by rapid thawing in a water bath set at 37 ^{o}C. Cells were then transferred to a 25 cm^2 culture flask already containing 10 ml pre-warmed medium. After 24 hours, media was replaced to minimise toxic effects of the cryoprotectant dimethyl sulphoxide (DMSO) and then maintained as per requirements per cell line.

2.1.2 Cell Line Requirements and Maintenance

2.1.2.1 Colorectal Cancer Cell Lines

SW480 cells (human colorectal adenocarcinoma) were cultured *in vitro* in 75 cm^2 non-vented culture flasks (Sarstedt Ltd., 68 Boston Road, Beaumont Leys, Leicester, LE4 1AW) in L-15 Leibovitz medium supplemented with 10% Fetal Calf Serum (FCS) (PAA Laboratories Ltd., Termare Close, Houndstone Business Park, Yeovil, Somerset, BA22 8YG) and 1% antibiotic solution [200 mM L-

glutamine/ 10,000 units/ml penicillin/ 10 mg/ml streptomycin solution (stabilised antibiotic solution)] and stored in an incubator set at 37 ^{o}C without CO_2.

HCT116 cells (human colorectal adenocarcinoma) and HT29 cells (human colorectal adenocarcinoma) were cultured *in vitro* in 75 cm^2 vented culture flasks (Sarstedt) in Dulbecco's Modified Eagle Medium (DMEM) (1 x) high glucose medium (PAA) supplemented with 10% FCS and 1% antibiotic solution [200 mM L-glutamine/ 10,000 units/ml penicillin/ 10 mg/ml streptomycin solution (stabilised antibiotic solution)] and stored in an incubator set at 37 ^{o}C with CO_2.

2.1.2.2 Breast Cancer Cell Lines

MDA231-MB cells (human breast carcinoma) were cultured *in vitro* in 75 cm^2 vented culture flasks in Dulbecco's Modified Eagle Medium (DMEM) (1 x) high glucose medium supplemented with 10% FCS and 1% antibiotic solution [200 mM L-glutamine/ 10,000 units/ml penicillin/ 10 mg/ml streptomycin solution (stabilised antibiotic solution)] and stored in an incubator set at 37 ^{o}C with CO_2.

MCF7 cells (human breast carcinoma) were cultured *in vitro* in 75 cm^2 vented culture flasks in Modified Eagle Medium (MEM) supplemented with 10% Fetal Calf Serum and 1% antibiotic solution [200 mM L-glutamine/ 10,000 units/ml penicillin/ 10 mg/ml streptomycin solution (stabilised antibiotic solution)] and stored in an incubator set at 37 ^{o}C with CO_2.

2.1.2.3 Glioma Cell Line

U373MG cells (human astrocytoma, grade III) were cultured *in vitro* in 75 cm^2 vented culture flasks in Dulbecco's Modified Eagle Medium (DMEM) (1 x) high glucose medium supplemented with 10% Fetal Calf Serum and 1% antibiotic solution [200 mM L-glutamine/ 10,000 units/ml penicillin/ 10 mg/ml streptomycin solution (stabilised antibiotic solution)] and stored in an incubator set at 37 ^{o}C with CO_2.

2.1.3 Subculture of Cell Lines

When cells reached 80 – 90% confluence they were subcultured using the following method. Media was removed by aspiration and cells were gently washed with 4 mls of trypsin EDTA solution (1 x containing 0.5 g/l EDTA.4Na in Hank's Balanced salt solution with phenol red) to remove any remaining serum which would inhibit the action of trypsin EDTA and prevent the detachment of cells. This 4 mls of trypsin EDTA was then removed by aspiration and a further 2 mls of trypsin was added to the flask which was subsequently placed in an incubator at 37 ^{o}C for approximately 5 minutes until the cells had detached and this was checked under the microscope. Eight mls of fresh pre-warmed medium was then added to the trypsinised cells in order to inactivate the trypsin. The cells were re-suspended and the cell suspension was divided into further flasks.

2.1.4 Liquid Nitrogen Storage of Cell Lines

Cell line stocks were maintained in liquid nitrogen storage (-180 ^{o}C). Cells were subcultured as described above, transferred to a sterile 10 ml universal tube and centrifuged at 1000 rpm for 2 minutes. The supernatant was carefully removed and the pellet was re-suspended in 90% (v/v) FCS and 10% (v/v) DMSO. Cells were aliquoted into 1.5 ml cryovials (Sarstedt) and placed in a polystyrene box and stored at -80 ^{o}C to slowly freeze. After 24 hours the cells were transferred to liquid nitrogen for long term storage.

2.1.5 Cell Viability Assay

Cell viability was measured by the 3-(4,5-dimethylthazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) conversion assay (Carmichael *et al.,* 1987). Briefly, cells were subcultured as described in 2.1.2 and quantified using a modified Neubauer haemocytometer. 10 µl of the subcultured cell suspension was pipetted into the haemocytometer chamber. The haemocytometer was viewed under a microscope and the numbers of cells occupying the four large corner squares and the central region of the chamber were counted and an average was calculated to determine the number of cells per ml. Cells were then seeded into 96 well plates (Sarstedt) at a concentration of 10^4 cells per 200 µl per well and the 96 well plate was covered with a plate seal (MP Biomedicals, Ohio, USA). For sterility, a new plate seal was used to replace one each time one was removed. After 24 hours of seeding, the medium was replaced with medium containing the drug treatment

at the required concentration or with diluent (either absolute ethanol or Tris-HCl buffer in order to determine any effects of the diluent) and incubated for the required time period. The medium was then removed and cells were incubated with 300 µl of medium containing MTT [0.5 mg/ml (w/v)] per well for 3 hours at 37 ^{o}C. The insoluble formazan product was solubilised with 200 µl DMSO per well. MTT conversion was determined by colorimetric analysis at 540 nm using a plate reader (Labsystems Multiskan MS). Results were calculated from the average of three independent experiments each performed in quintuplicate and are reported as the percentage of treated cells relative to the cells treated with medium alone using the equation:

% Cell Viability = (absorbance of treated cells/mean absorbance of untreated cells) x (100/1)

Statistical analysis of data was performed using GraphPad Prism 5 (version 5.01, GraphPad Software Inc., San Diego, CA 92130, USA).

2.1.6 Cell Treatments – Preparation of Aspirin and Salicylic Acid Stocks

0.5 M stocks of aspirin (Sigma, UK) and salicylic acid (Sigma, UK) were prepared fresh before each treatment. Calculated amounts of these stock solutions were then added to cell medium to provide the appropriate concentration of medium which was then used to treat cells.

Aspirin and salicylic acid were either dissolved in absolute alcohol to provide a 0.5 M stock solution or dissolved in 1 M Tris-HCl (pH 7.5) with 6 M NaOH to a stock concentration of 0.5 M and pH adjusted to 7.2 with 4 M HCl.

2.2 Molecular Assays

2.2.1 RNA Extraction and Quantification

RNA was extracted from cells using a RNAqueous® – 4PCR kit for isolation of DNA-free RNA utilising DNase I treatment (Ambion, Austin, TX). RNA extraction was carried out in a Telstar hood which had undergone UV irradiation prior to use. Cells were grown in 75 cm^2 flasks and were either treated or untreated with the required drug. Medium was aspirated from the flasks and the cells were then washed with ice cold phosphate buffered saline (PBS). 500 µl of lysis/binding (guanidinium) solution was added to the flask and cells were scraped from the flask with cell

scrapers to form a cell suspension which was transferred to a 1.5 ml Eppendorf tube and vortexed for approximately 20 seconds to homogenise the sample. 500 µl of 64% ethanol was then added to the sample and the sample was mixed by inversion. A silica-based filter cartridge (provided in the kit) which selectively and quantitatively binds mRNA was inserted into an elution tube to form a filter unit and 700 µl of cell suspension was added to the assembled filter unit. The filter unit was spun in a microfuge at 10,000 rpm for 2 - 3 minutes until the lysate/ethanol mixture had passed through the filter and the flow-through was discarded. The next stages involved washing the filter to remove traces of DNA, protein and any other contaminants. 700 µl of Wash Solution 1 was added to the filter unit and spun in microfuge at 10,000 rpm until all the wash solution had passed through the filter. The flow-through was discarded and the filter was then washed twice with 500 µl of Wash Solution 2/3 which was added to the filter unit and centrifuged at 10,000 rpm until the wash solution had passed through the filter. The flow through was discarded each time. The filter cartridge was then removed from the filter unit and placed inside a fresh elution tube. Elution Solution (nuclease-free water containing EDTA to chelate heavy metals) pre-heated to 80 ^{o}C, was added to the centre of the filter and the filter unit was centrifuged for 30 seconds at 10,000 rpm resulting in eluted RNA being collected in elution tube. To avoid genomic DNA contamination, RNA was treated with DNase. 0.1 volume of 10 x DNase I buffer and 1 µl of DNase I was added to eluted RNA and incubated for 30 minutes at 37 ^{o}C. 0.1 volume of DNase Inactivation Reagent was then added to the mixture, and incubated for 2 minutes at room temperature, flicking the tube occasionally to allow mixing. The sample was then centrifuged at 10,000 rpm for 1 minute to pellet DNase Inactivation Reagent and the supernatant containing the RNA was transferred to a fresh 0.5 µl Eppendorf tube and quantified before use.

Total RNA concentration was then quantified using a Pharmacia GeneQuant II spectrophotometer. The RNA concentration (µg/ml) and RNA purity (A_{260}/A_{280} ratio) was measured. Sample concentrations were recorded and any samples having A_{260}/A_{280} ratios of below 1.5 were discarded.

2.2.2 First Strand cDNA Synthesis

cDNA was prepared using a 1st strand cDNA synthesis kit for RT-PCR (AMV) (Roche, UK) using 500 ng RNA on a PTC-100^{TM} Programmable thermal controller (MJ Research, Inc). The template was initially incubated with 2 µl Oligo-p(dT)$_{15}$ primer at 70 ^{o}C for 5 minutes and then snap-cooled on ice for 5 minutes. The final reaction was carried out in a final volume of 20 µl including 2 µl of 10

x Reaction Buffer, 4 µl 25 mM $MgCl_2$, 2 µl Deoxynucleotide mix (dNTPs), 1 µl RNase Inhibitor and 0.8 µl AMV Reverse Transcriptase for 5 minutes at 25 ^{o}C then at 42 ^{o}C for 60 minutes. The reaction was stopped by heating at 70 ^{o}C for 15 minutes and cDNAs were then used in subsequent PCR techniques or stored at -20 ^{o}C until use.

2.2.3 Semi-Quantitative Polymerase Chain Reaction (PCR)

Semi-Quantitative PCR was carried out using Roche Amplitaq Gold Kit on a PTC-100^{TM} Programmable thermal controller (MJ Research Inc). 5 µl of cDNA was amplified in a final volume of 50 µl containing 1 nM of each forward and reverse primer (Table 2.1), 5 µl 10 x PCR buffer, 5 µl 2 mM dNTP, 5 µl 25 mM $MgCl_2$ and 0.5U AmpliTaq DNA polymerase. Primers were obtained from Sigma Genosys.

Table 2.1. Primers used in Semi-Quantitative PCR

Primer	Sequence (5' – 3')
MBD4 Forward	TGCCGCCAAGTAGTAGTTC
MBD4 Reverse	TGGGATTCCTTTCAAAATAGTC
Actin Forward	ATAGCACAGCCTGGATAGCAACGTAC
Actin Reverse	CACCTTCTACAATGAGCTGCGTGTG

Table 2.2. Cycling Parameters for Semi-Quantitative PCR

Step	Temperature	Time
1	95 ^{o}C	12 minutes
2 (28 cycles)	95 ^{o}C	1 minute
	55 ^{o}C	1 minute
	72 ^{o}C	90 seconds
3	72 ^{o}C	5 minutes
4	32 ^{o}C	15 minutes

2.2.4 PCR Gel Electrophoresis

Semi-Quantitative PCR products were electrophoresed on a 3% Agarose gel (1.5 g Agarose in 50 ml 1 x Tris-Borate-EDTA (TBE) buffer) plus 5 µl (1% (v/v)) ethidium bromide. 7 µl of product and 3 µl of loading dye (Abgene, UK) were mixed and loaded onto the gel and ran at 50 volts for 60 minutes and visualised using ultraviolet (UV) light. 100 base pair marker (Abgene, UK) was used to indicate size of bands. Gels were visualised using a Biovision UV gel imager (Perceptics, Knoxville, TN) and 'Grabber' software was employed (Perceptics, Knoxville, TN).

2.2.5 geNorm Analysis

For normalising Quantitative Real-Time PCR data, suitable reference genes were selected using the geNorm™ Housekeeping Gene Selection Kit (PrimerDesign Ltd., Southampton, UK). 12 candidate reference genes were analysed for suitability for use as reference genes for this particular experimental model.

Lyophilised primers for the following reference genes *ACTB, GAPDH, UBC, B2M, YWHAZ, SF3A1, 18S, CYC1, EIF4A2, SDHA, TOP1 and ATP5B* were provided in the kit and re-suspended in RNAse/DNAse free water also provided in kit.

RNA from ten 1 mM aspirin treated SW480 samples and ten untreated SW480 samples was extracted as previously described in 2.2.1 and 132 µl of cDNA for each sample at a concentration of 5 ng/µl was prepared using first strand cDNA synthesis as described previously.

Table 2.3. Components of mix for geNorm analysis.

Component	**1 reaction**
Resuspended primer mix (300 nM)	1 µl
PrimerDesign Precision™ 2 x qPCR Mastermix	10 µl
RNAse/DNAse free water	4 µl
Final Volume	15 µl

5 µl of each cDNA sample was then pipetted into a single well of a 96 well plate and 15 µl of the mix was added to each cDNA sample. Each reference gene was run on the same 96 well plate and

all cDNA samples were run in duplicate wells. Plates were then run on the iCycler Real-Time PCR detection system (Bio-Rad Laboratories Inc, UK) using the amplification protocol in Table 2.4.

Table 2.4. Amplification protocol for geNorm analysis.

Step	Temperature	Time
Enzyme activation	95 ^{o}C	10 minutes
Denaturation	95 ^{o}C	15 seconds
Data Collection (50 cycles)	60 ^{o}C	60 seconds

Data was analysed using templates and data analysis software provided by the supplier to determine the most stably expressed reference genes and how many reference genes were needed for optimal normalisation of data.

2.2.6 Quantitative Real-Time PCR

Quantitative Real-Time PCR was carried out using the iCycler Real-Time PCR detection system (Bio-Rad, UK) in 96 well microtitre plates (Abgene, UK). RNA from samples was extracted and reverse transcribed as previously described. cDNA was diluted 1:10 in nuclease-free water prior to analysis. The mixes made for each primer set are shown in Table 2.5.

Table 2.5. Components of mix used for Quantitative Real-Time PCR

Component	1 reaction using in house primers (µl)	1 reaction using prevalidated primer mix (µl)
PrimerDesign Precision™ 2 x qPCR Mastermix	12.5	12.5
Water	5.5	6.25
Forward primer	1	0
Reverse Primer	1	0
Prevalidated Primer Mix	0	1.25
Final volume	20	20

5 µl of cDNA sample was then pipette into a single well of a 96 well plate and 20 µl of the mix was added to each cDNA sample. Each primer set was run on a separate 96 well plate and each cDNA sample was run in triplicate. 96 well plates were capped using plastic strips (Abgene, UK) before being sealed using plate sealers (Abgene, UK) to prevent sample evaporation and then run on the iCycler Real-Time PCR detection system (Bio-Rad, UK).

Primers were either designed in-house or were prevalidated primers from PrimerDesign Ltd, UK. Prevalidated forward and reverse primers were provided in a single mix. Sequences are given in Table 2.6.

Table 2.6. Primer sequences and annealing temperatures used for Quantitative Real-Time PCR

Primer	Sequence (5' – 3')	Annealing Temperature
MBD4 Forward (In-house)	TGCCGCCAAGTAGTAGTTC	55 ^{o}C
MBD4 Reverse (In-house)	TGGGATTCCTTTCAAAATAGTC	55 ^{o}C
BRCA1 Forward (Prevalidated)	AGAGAATCCCAGGACAGAAAGA	57 ^{o}C
BRCA1 Reverse (Prevalidated)	ACCACAGAAGCACCACACA	57 ^{o}C
ATR Forward (Prevalidated)	CACCACCAGACAGCCTACA	56 ^{o}C
ATR Reverse (Prevalidated)	GAGCCACTTGCCCTTTCC	56 ^{o}C
GADD45α Forward (Prevalidated)	TACTCCCTACACTGATGCAAG	55 ^{o}C
GADD45α Reverse (Prevalidated)	CATCTGCAAAGTCATCTATCTCC	55 ^{o}C
XRCC3 Forward (Prevalidated)	TGGACCAGACTTGAAGAGACT	56 ^{o}C
XRCC3 Reverse (Prevalidated)	GTGCTGTAAGGATGCTGCTT	55 ^{o}C

Cycling parameters are shown in Table 2.7 and include melt curve data collection for analysis.

Table 2.7. Cycling parameters for Quantitative Real-Time PCR

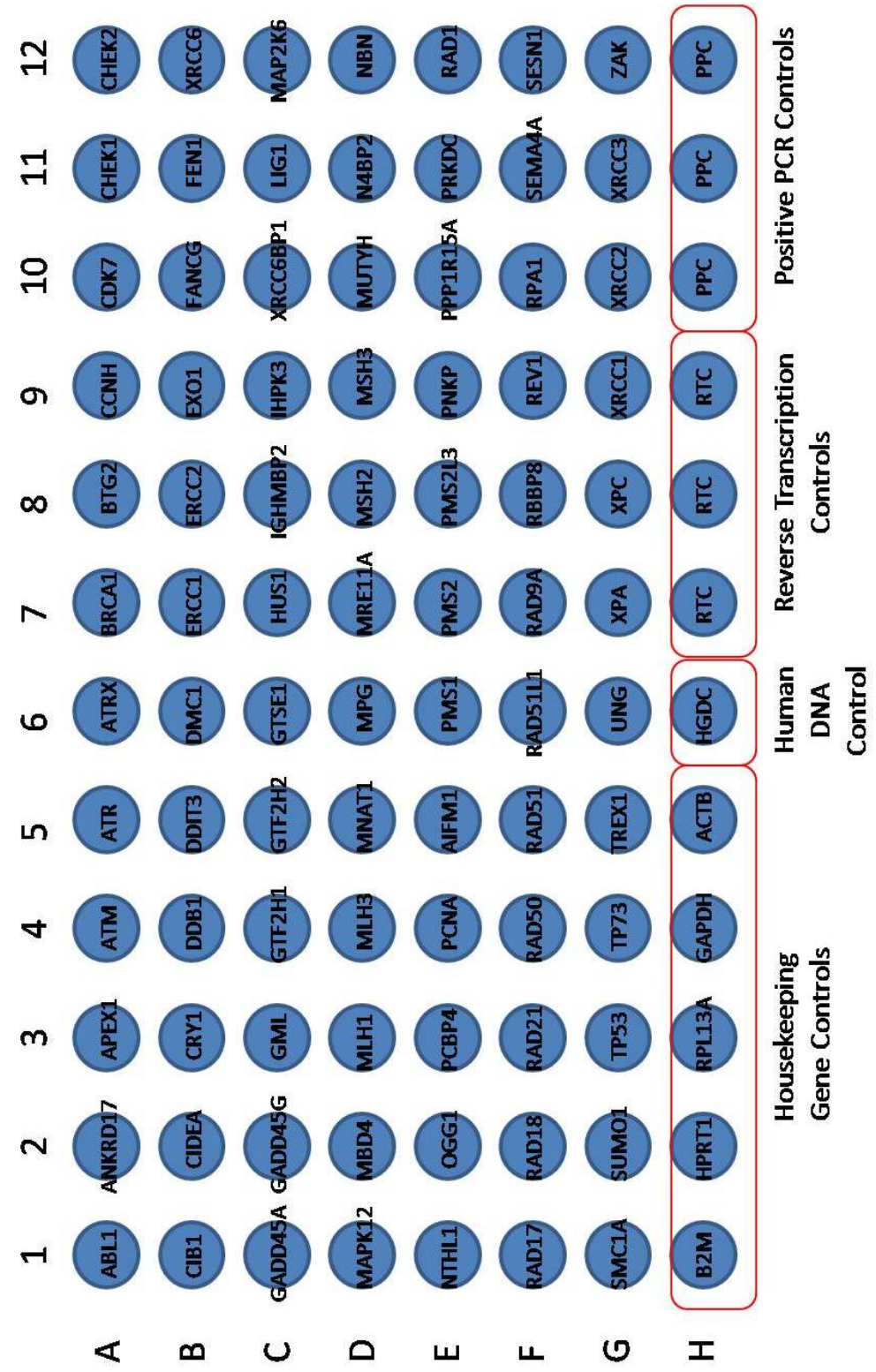
Step	Temperature	Time
1	95 ^{o}C	15 minutes
2 (40 cycles)	95 ^{o}C	30 seconds
	55 ^{o}C*	30 seconds
	72 ^{o}C	30 seconds
Data collection and real-time analysis enabled ***= this annealing temperature was altered according to recommended temperature for each individual primer set**		
3	55 ^{o}C	1 minute
4 (80 cycles)	55 ^{o}C	10 seconds
Increase in setpoint temperature after the second cycle by 0.5 ^{o}C for melt curve data collection and analysis		

2.2.7 RT^{2} Profiler^{TM} PCR Array System

For PCR array analysis, the Human DNA Damage Signalling Pathways RT^{2} Profiler^{TM} array (SuperArray Biosciences Corporation, Frederick, MD) of 84 genes related to the DNA damage signalling pathways, five reference genes, RNA quality control, reverse transcription and PCR efficiency controls were utilised on a Bio-Rad iCycler.

Each well of the 96 well array plate contained lypholised primers for a gene involved in human DNA damage signalling pathways as indicated in Figure 2.1.

Figure 2.1. PCR Array layout



500 ng total RNA was reverse transcribed using the first strand cDNA synthesis kit for RT-PCR (AMV) as previously described in 2.2.2. The resulting cDNA was diluted 1 in 10 in nuclease-free water and added to SYBR Green Fluorescein Master Mix (PrimerDesign Ltd, Southampton, UK) to form Experimental Cocktail 1 mix (shown in Table 2.8) which was subsequently aliquoted to each well of the PCR array except wells H6 – H12.

Table 2.8. Experimental Cocktail 1 for RT Profiler™ PCR array

Component	Volume
PrimerDesign Precision™ 2 x qPCR Mastermix	1225 µl
Water	1127 µl
cDNA	98 µl
Total	2450 µl

For the control wells (H6 – H12), a 10-fold serial dilution (shown in Table 2.10) of Experimental Cocktail 2 (shown in Table 2.9) was prepared.

Table 2.9. Experimental Cocktail 2 for RT Profiler™ PCR array

Component	Volume
PrimerDesign Precision™ 2 x qPCR Mastermix	100 µl
Water	100 µl
Total	200 µl

Table 2.10. Serial dilution preparation for RT Profiler™ PCR array

For Well	H6	H7	H8	H9	H10
Experimental Cocktail 2	27 µl	27 µl	27 µl	27 µl	27 µl
Material used for previous well	3 µl	3 µl	3 µl	3 µl	3 µl

For well H11 1 µl of a 1:100 dilution of the original RNA was pipetted. For well H12 25 µl of Experimental Cocktail 2 was pipetted.

Five untreated samples and five treated samples were run on individual plates and data was analyzed with the Excel-based PCR Array Data Analysis Template downloaded from the SuperArray website (www.sabiosciences.com) and normalised to the expression level of reference control genes provided on the arrays.

A list of genes on the array are given in Table A1 in Appendix 1.

The cycling parameters for the RT^2 Profiler™ PCR array are shown in Table 2.11.

Table 2.11. Cycling parameters for RT Profiler™ PCR array

Step	Temperature	Time
1	95 °C	10 minutes
2 (40 cycles)	95 °C	15 seconds
	60 °C	1 minute
Data collection and real-time analysis enabled		
3	95 °C	1 minute
4	55 °C	1 minute
5 (80 cycles)	55 °C	10 seconds
Increase in setpoint temperature after the seconds cycle by 0.5 °C for melt curve data collection and analysis		

2.3 Proteomic Analysis

2.3.1 Protein Extraction

For protein extraction, cells were washed with ice-cold PBS, then protein was extracted using protein extraction buffer [20 mM Tris/150 mM NaCl/1 mM EDTA/1% (v/v) Triton X, including protease inhibitors (Complete Mini Protease Inhibitor Cocktail Tablets, Roche)] on ice for 1 hour and then centrifuged at 13,000 *g* at 4 °C to remove cell fragments. Samples were then quantified as described in 2.3.2 and stored at -20 °C.

For samples analysed at KuDOS Pharmaceuticals, (Cambridge, UK) samples were extracted using RIPA buffer (10% (v/v) NP-40/10% (v/v) Na-deoxycholate/100 mM EDTA in Tris buffer pH 7.4

including Protease inhibitor cocktail tablet (Roche), Phosphatase inhibitor cocktail 1, Phosphatase inhibitor cocktail 2 and 200 mM PMSF). Cell pellets were thawed on ice, re-suspended in 3 bed volumes of extraction buffer and incubated on ice for 20 minutes. Whole cell extracts were obtained by centrifuging samples at 13,000 rpm at 4 ^{o}C for 15 minutes. Supernatants were transferred to fresh Eppendorf tubes and samples were quantified using BCA Protein Assay kit (Pierce**,** Northumberland, UK).

2.3.2 Quantification of Protein Concentration

Protein concentrations were quantified using a DC Protein Assay (Bio-Rad, UK). This kit uses a modified Lowry reaction to create a colorimetric reaction which was measured at 690 nm using a plate reader (Labsystems Multiskan MS). The protein concentrations of the unknown samples were determined by comparison of the absorbance of the unknown samples to the standard curve prepared using Bovine Serum Albumin (BSA) protein standards. All samples were quantified in triplicate and averages taken for quantification purposes. Once quantified, the protein was re-suspended in an appropriate amount of 2 x Laemelli sample buffer [4% (w/v) SDS, 20% (v/v) glycerol, 10% (v/v) 2-mercaptoethanol, 0.004% (w/v) bromophenol blue, 0.125 M Tris.HCl pH 6.8] which had been calculated to allow for equal loading of the samples for SDS-PAGE electrophoresis. Protein was denatured by boiling at 100 ^{o}C for 2 minutes and then stored at -20 ^{o}C until use.

2.3.3 Protein Precipitation

Where necessary, protein was precipitated from samples according to the Wessel and Flugge method (Wessel and Flugge, 1984). Briefly, one volume of sample, four volumes of methanol, one volume of chloroform and three volumes of distilled water were mixed by inversion and then centrifuged at 10,000 rpm for 5 minutes. The supernatant was carefully removed and three volumes of methanol were then added to the remaining sample and centrifuged at 10,000 rpm for 5 minutes. The supernatant was then discarded and the protein was allowed to air dry for 30 minutes. The protein was then re-suspended in an appropriate amount of 2 x Laemelli sample buffer [4% (w/v) SDS, 20% (v/v) glycerol, 10% (v/v) 2-mercaptoethanol, 0.004% (w/v) bromophenol blue, 0.125 M Tris HCl pH 6.8] which had been calculated to allow for loading of the sample for SDS-PAGE electrophoresis. Protein was denatured by boiling at 100 ^{o}C for 2 minutes and stored at -20 ^{o}C until use.

2.3.4 Sodium Dodecyl Sulphate - Polyacrylamide Gel Electrophoresis (SDS-PAGE)

For the purpose of protein sample analysis, discontinuous sodium dodecyl sulphate polyacrylamide electrophoresis (SDS-PAGE) was used. Denatured protein samples were loaded into wells in a 4% polyacrylamide stacking gel and the proteins in the samples were separated by SDS-PAGE on a polyacrylamide resolving gel in 1 x Tris-Glycine-SDS Running Buffer (Sigma, UK) at 180 volts for approximately 60 minutes (or until dye front had reached bottom of the gel) at room temperature using Bio-Rad Protean II gel casting equipment. Molecular weight markers were loaded alongside samples to allow for protein molecular weight analysis.

Molecular Weight Markers used were SeeBlue® Plus2 Pre-Stained Standard (Invitrogen), Biorad prestained SDS-PAGE standards, Broad Range marker (Bio-Rad), HiMarkTM Pre-Stained HMW Protein Standard (Invitrogen).

Depending on the molecular weight of the protein to be analysed, the separating gel was either 6%, 12% or 15% acrylamide [from 30% stock solution (37.5:1 acrylamide:bis acrylamide)] (Bio-Rad, UK). Gels were prepared and allowed to mature by storing overnight at 4 ^{o}C before use. The compositions are given in Table 2.12.

Table 2.12. Compositions of SDS-PAGE gels

Component	6% Resolving Gel (ml)	12% Resolving Gel (ml)	15% Resolving Gel (ml)	Stacking Gel (ml)
Lower Gel 4x Buffer [0.5M Tris.HCl, 0.4% SDS to pH 6.8 with HCl]	2.5	2.5	2.5	0
Upper Gel 4 x Buffer [1.5M Tris.HCl, 0.4% SDS to pH 8.8 with HCl)	0	0	0	2.5
Distilled water	5.9	4.4	3.65	6.6
40% Acrylamide/Bis solution 37.5:1 (2.6% C) solution	1.5	3.0	3.75	0.8
10% Ammonium persulphate (APS)	50 µl	50 µl	50 µl	100 µl
N,N,N',N,-tetramethylethylenediamine (TEMED)	5 µl	5 µl	5 µl	10 µl

For BRCA1 and ATR protein analysis, samples were analysed at KuDOS Pharmaceuticals Ltd, (Cambridge, UK) using pre-cast 3 – 8% Tris-Acetate gels (Invitrogen). Samples were run in Tris Acetate running buffer at 120V until the dye front had reached the bottom of gel.

Gels were either Coomassie Blue stained, silver stained or transferred to a nitrocellulose membrane for immunoblotting.

2.3.5 Immunoblotting

SDS-PAGE resolved proteins were transferred at 100 volts for 1 hour onto a nitrocellulose membrane (Bio-Rad) in 1x Tris-Glycine Transfer Buffer with methanol [100 ml Tris-Glycine buffer (Sigma, UK) made up to 1000 ml with 700 ml distilled water and 200 ml methanol] at 4^{o}C. Efficient transfer was monitored using Ponceau S Stain [0.1% (w/v) Ponceau S Stain in 5% (v/v) acetic acid]. To block non-specific binding, the membrane was incubated in blocking solution [5% (w/v) BSA or 5% (w/v) milk powder (Marvel)/0.2% (v/v) Tween 20 in TBS] either overnight at 4 ^{o}C or for 1 hour at room temperature. The membrane was then incubated with primary antibody for either 1 hour at room temperature or overnight at 4 ^{o}C. Primary antibody was diluted in blocking solution to the required concentration. The membrane was then washed with washing solution [0.2% (v/v) Tween 20 in TBS] for 1 hour, with the wash solution replaced every 10 minutes. The membrane was then incubated with the appropriate HRP-conjugated secondary antibody in blocking solution for 1 hour at room temperature. The membrane was again washed using wash solution for 1 hour and then incubated with Enhanced Chemiluminescence (ECL Plus^{TM} detection kit) (GE Healthcare) and analysed using a Storm 840 phosphorimager with Imagequant software (GE Healthcare). Although samples had been quantified to ensure equal concentrations of samples were loaded for comparison studies, to ensure equal band loading, samples were also probed for beta-tubulin protein expression using a monoclonal anti-beta tubulin antibody.

For BRCA1 and ATR gels, resolved proteins were transferred onto PVDF membrane (which had been equilibrated in 100% methanol and the equilibrated in Transfer buffer for 15 minutes prior to transfer) at 350 mA for 1 hour. Before blocking, PVDF membrane was re-hydrated in 100% methanol and washed briefly in PBS. The membrane was blocked in blocking buffer [4% (w/v) Marvel in PBS] for 1 hour at room temperature and incubated with primary antibody (diluted in blocking buffer) overnight at 4 ^{o}C. The membrane was then washed 3 times in wash solution [0.1% (v/v) Tween 20 in PBS] for 5 minutes each time and incubated with secondary antibody

[diluted in 5% (w/v) Marvel/0.1% (v/v) Tween 20 in TBS] for 1 hour at room temperature. The membrane was again washed 3 times using wash solution (5 minutes) and then incubated with Western Lightning™ Plus-ECL oxidising reagent plus, enhanced Luminol reagent plus (New England Biosciences). The membrane was then analysed using Intelligent Dark box, LAS-3000 (Fujifilm) and Image Reader, LAS-3000 with an exposure time of 20 minutes.

Tables 2.13 and 2.14 show the antibodies used for immunoblotting.

Table 2.13. Primary antibodies used for immunoblotting

Primary Antibody	Dilution	Company
Anti-HIS	1:2000	Sigma
Anti-GST	1:2000	Sigma (#A7340)
Rabbit Polyclonal anti-BRCA1	1:1000	New England Biolabs (#9010)
Rabbit Polyclonal anti-ATR	1:100	New England Biolabs (#2790)
Rabbit Polyclonal anti-GADD45α	1:250	Santa Cruz (#SC-797)
Rabbit Polyclonal anti-XRCC3	1:5000	Abcam (#AB6494)
Mouse anti-alpha tubulin	1:5000	Sigma (#T5168)
Anti-beta tubulin	1:5000	Abcam
Mouse anti-alpha tubulin	1:5000	Abcam (#AB7291)

Table 2.14. Secondary antibodies used for immunoblotting

Secondary Antibody	Dilution	Company
Anti-Rabbit HRP conjugated	1:1000	Oncogene
Anti-Mouse HRP conjugated	1:10,000	Sigma (#A0168)
Goat anti-mouse IgG HRP conjugated	1:10,000	Pierce (#31430)
Anti-rabbit	1:10,000	Pierce (#31460)

2.3.6 Staining of SDS-PAGE gels

2.3.6.1 Coomassie Blue Staining

For Coomassie Blue staining, once protein samples had been separated by SDS-PAGE, the gel was obtained and the stacking gel was removed. The gel was then washed three times in distilled water for 5 minutes each time. The gel was then immersed in Coomassie stain [50% (v/v) methanol, 10% (v/v) acetic acid, 0.25% (w/v) brilliant blue R] for 20 minutes at room temperature with gentle agitation. The Coomassie stain solution was then poured off and the gel was immersed in destain solution [10% (v/v) methanol, 5% (v/v) acetic acid] overnight at room temperature with gentle agitation. Gels were stored in ultrapure water.

2.3.6.2 Silver Staining

For Silver Staining, after SDS-PAGE electrophoresis, the gel was stained using the ProteoSilver Plus Silver Stain kit (Sigma, UK). The gel was incubated with Fixing Solution (50% ethanol, 10% acetic acid, 40% ultrapure water) for 40 minutes. The gel was then incubated with 30% ethanol for 10 minutes and washed in ultrapure water for 10 minutes. Sensitizer solution was used to sensitise the gel for 10 minutes. The gel was then washed twice with ultrapure water for 10 minutes each time. The gel was then equilibrated with Silver Solution for 10 minutes and then washed with ultrapure water for 1 minute. The gel was developed with Developer Solution until the desired

staining intensity was achieved. ProteoSilver Stop Solution was added to stop the developing reaction. Gels were stored in ultrapure water.

2.3.7 Dot Blotting

For analysis of presence of his-tagged protein during protein purification, 0.5 µl of protein sample was dotted onto nitrocellulose membrane (Bio-Rad) and allowed to air dry. The membrane was then blocked in buffer [5% (w/v) BSA/0.2% (v/v) Tween 20 in PBS] overnight at 4 ^{o}C. The membrane was then incubated with anti-His HRP antibody (1:2000 dilution) for 30 minutes at room temperature and then washed with wash solution [0.2% Tween 20 in PBS] for 30 minutes, with the wash solution replaced every 10 minutes. The membrane was then washed in PBS only to remove any excess Tween 20. The membrane was incubated with Enhanced Chemiluminescence (ECL Plus^{TM} detection kit) (GE Healthcare) and analysed using a Storm 840 phosphorimager with Imagequant software (GE Healthcare).

For analysis of binding of recombinant MLH1 (GST-tagged) (Abnova, Taiwan) to sMBD4 the same methodology was used and either MLH1 was dotted onto nitrocellulose membrane and incubated with sMBD4 for 1 hour at room temperature before being analysed with anti-His HRP antibody or sMBD4 was dotted onto nitrocellulose membrane and incubated with MLH1 for 1 hour at room temperature and analysed with anti-GST HRP antibody.

2.3.8 Immunoprecipitation

To allow the detection of binding of recombinant MLH1 (Abnova, Taiwan) to sMBD4 by immunoprecipitation, an EZview^{TM} Red HIS-select^{TM} HC Nickel Affinity gel (Sigma, UK) was used. Beads were equilibrated by washing 3 times with ice cold lysis buffer [20% (v/v) glycerol, 0.1% (v/v) Tween 20 in PBS] for 5 minutes each time. Beads were centrifuged at 10,000 rpm for 30 seconds to pellet and supernatant was removed each time. Beads were then incubated with protein (either sMBD4 only, MLH1 only or both sMBD4 and MLH1) for 2 hours at 4 ^{o}C on a rotating wheel. Samples were then centrifuged at 10,000 rpm for 30 seconds and the supernatant was removed. Samples were then vortexed with lysis buffer, re-suspended in 2 x Laemmli sample buffer [4% (w/v) SDS, 20% (v/v) glycerol, 10% (v/v) 2-mercaptoethanol, 0.004% (w/v) bromophenol blue, 0.125 M Tris.HCl pH 6.8] and then boiled for 5 minutes to elute the protein samples from the beads. Samples were centrifuged at 10,000 rpm for 30 seconds to

pellet beads and the resulting supernatant was transferred to a fresh Eppendorf tube and stored at -20 ^{o}C until analysis by SDS-PAGE and western blotting.

2.3.9 Immunofluorescence

At approximately 50 – 60% confluency, flasks were subcultured using Trypsin-EDTA (Sigma) into a six well plate (Sarstedt) containing a sterile coverslip (SLS) and incubated for 24 hours at 37 ^{o}C. After 24 hours, medium was aspirated from each well and replaced with untreated medium, 1 mM aspirin or 1 mM salicylic acid and then incubated for 48 hours at 37 ^{o}C. After incubation, medium was aspirated from the wells and coverslips were washed with 2 mls ice cold PBS to remove excess medium. The coverslips were then fixed with ice cold methanol for 5 minutes followed by ice cold acetone for 5 minutes then washed in PBS three times for 10 minutes each. The PBS was aspirated off and replaced with a blocking buffer [3% (w/v) BSA/0.2% (v/v) Tween 20 in PBS] for 1 hour to prevent non-specific binding. The blocking agent was washed off with PBS and the primary antibody diluted in blocking buffer was placed on the coverslip. The primary antibody was left to incubate at room temperature and after 1 hour was washed off in PBS and the coverslips were washed in PBS three times for 10 minutes. This was replaced by a fluorescent tag conjugated (FITC or Texas Red) secondary antibody diluted in blocking agent and was left to incubate for 1 hour at room temperature. Post incubation the antibody was washed off with PBS three times for 10 minutes. The coverslips were then mounted onto slides with mounting medium (Dako). Images were obtained using a Zeiss LSM 510 confocal microscope (Carl Zeiss, US).

Tables 2.15 and 2.16 show antibodies used for immunofluorescence analysis.

Table 2.15. Primary antibodies used for immunofluorescence analysis

Primary Antibodies	Dilution	Company
Mouse monoclonal anti-BRCA1	1:100	Abcam (#AB81-100)
Rabbit polyclonal anti-ATR	1:100	Abcam (#AB2905-100)
Rabbit polyclonal anti-GADD45α	1:250	Santa Cruz (#SC-797)
Rabbit polyclonal anti-XRCC3	1:5000	Abcam (#AB6494)

Table 2.16. Secondary antibodies used for immunofluorescence analysis

Secondary Antibodies	Dilution	Company
Anti-Rabbit FITC conjugated	1:200	Santa Cruz (#SC-2090)
Anti-Mouse Texas Red conjugated	1:200	Santa Cruz (#SC-2785)

2.3.10 Immunohistochemistry

At approximately 50 – 60% confluency, flasks were subcultured using Trypsin-EDTA (Sigma) into a six well plate (Sarstedt) containing a sterile coverslip (SLS) and incubated for 24 hours at 37 ^{o}C. After 24 hours, medium was aspirated from each well and replaced with untreated medium, 1 mM aspirin or 1 mM salicylic acid and then incubated for 48 hours at 37 ^{o}C. After incubation, medium was aspirated from the wells and coverslips were washed with 2 mls ice cold PBS to remove excess medium. The coverslips were then fixed with ice cold methanol for 5 minutes followed by ice cold acetone for 5 minutes then washed in PBS three times for 10 minutes each. The PBS was aspirated off and replaced with a blocking buffer [3% (w/v) BSA/0.2% (v/v) Tween 20 in PBS] for 1 hour to prevent non-specific binding. The blocking agent was washed off with PBS and the primary antibody diluted in blocking buffer was placed on the coverslip. The primary antibody was left to incubate at room temperature and after 1 hour was washed off in PBS and the coverslips were washed in PBS three times for 10 minutes. This was replaced by a HRP conjugated secondary antibody diluted in blocking agent and was left to incubate for 1 hour at

room temperature. Post incubation the antibody was washed off with PBS three times for 10 minutes. The coverslips were then incubated with 1-Step™ TMB substrate (Pierce) for 4 minutes before the reaction was stopped with distilled water. The coverslips were then mounted onto slides with mounting medium (Dako). Images were obtained using an inverted microscope and analysed using analytical digital photomicroscopy (ADP).

Tables 2.17 and 2.18 show the antibodies used for immunohistochemistry.

Table 2.17. Primary antibodies used for immunohistochemistry.

Primary antibodies	Dilution	Company
Rabbit polyclonal anti-XRCC3	1:5000	Abcam (#AB6494)

Table 2.18. Secondary antibodies used for immunohistochemistry

Secondary antibodies	Dilution	Company
Anti-Rabbit HRP conjugated	1:1000	Oncogene

2.4 Protein Expression and Purification

2.4.1 Growth of sMBD4 *E. coli* expression cultures

Escherichia Coli expression vectors were obtained from Dr Iain Nicholl – University of Wolverhampton [sMBD4 cDNA cloned into pQE30 expression vector (Qiagen) using strain SG13009[pREP] (Qiagen) as a host]. (See Appendix for map and sequence details).

LB medium [20 g Tryptone/10 g Log phase yeast extract/20 g NaCl] and LB agar [LB medium containing 15 g/L agar(#2)] were prepared and autoclaved. The LB Agar was allowed to cool to 50^{o}C and antibiotics were added (25 µg/ml kanomycin and 100 µg/ml ampicillin). LB Agar was then poured into Petri dishes and allowed to set at room temperature. *E.coli* cells were streaked onto an agar plate and incubated overnight at 37 ^{o}C. 50 ml cultures of LB medium with 25 µg/ml

kanomycin and 100 µl/ml ampicillin were started from single colonies and grown overnight at 37 ^{o}C shaking at 150 rpm. 750 ml cultures were inoculated the following morning and allowed to grow for 4 hours. Protein expression was induced with 1 mM IPTG, and cultures were allowed to grow for an additional 4 hours. The bacteria were pelleted by spinning at 4000 *g* for 2 hours at 2 ^{o}C. The cells were re-suspended in 50 ml PBS, centrifuged at 4000 *g* for 1 hour and stored at -80 ^{o}C.

2.4.2 Native Protein Purification of sMBD4

An *E.coli* pellet was thawed at room temperature and 2 ml Bugbuster (Novagen) and 1 mM PIC was added. The cells were then lysed in a Dounce homogenizer (5 strokes) and left at room temperature for 15 minutes. Eight mls of ice cold Native buffer [2 x PBS, 500 mM NaCl, 20 mM imidazole] was added to the sample and dounce homogenised (10 strokes) on ice. Cell debris and nuclei were pelleted (13,000 rpm for 30 minutes at 2 ^{o}C) and the supernatant was then dialysed twice in 500 mls Native buffer at 4 ^{o}C for 2.5 hours each time using SnakeSkin™ Dialysis Tubing (Pierce). The pellet was snap frozen in liquid nitrogen and stored at -80 ^{o}C for future analysis. The dialysed sample was then passed through a HisTrap™ HP column (GE Healthcare) using an ÄKTA Prime Plus using preprogrammed application template HISTAG PURIFICATION HISTRAP with Native buffer and Native Elution buffer [2 x PBS, 500 mM NaCl, 500 mM imidazole]. Buffers were filtered through sterile 0.45 µm filters before use.

2.4.3 Denaturing Protein Purification of sMBD4

Denaturing purification of sMBD4 was carried out as previously described (Owen *et al.,* 2007).
An *E.coli* pellet was thawed at room temperature and 2 ml Bugbuster (Novagen) and 1 mM Protease Inhibitor Cocktail was added. The cells were then vortexed and left at room temperature for 15 minutes. The cells were then lysed in a Dounce homogenizer (5 strokes) and 8 mls of denaturing buffer [25 mM Tris.HCl (pH 8), 8 M urea, 10 mM NaCl, 1 mM EDTA, 1 mM DTT] was added to the sample and dounce homogenised (10 strokes). The sample was then left for 1 hour at room temperature to dissolve protein. The sample was then dialysed for 2.5 hours in 500 ml denaturing buffer at room temperature using SnakeSkin™ Dialysis Tubing (Pierce). The dialysed sample was then centrifuged (13,000 rpm for 30 minutes at 20 ^{o}C). The supernatant was taken for further analysis (fractionation over HiTrap™ Q column and HisTrap™ column) and the pellet was frozen at -80 ^{o}C.

2.4.4 HiTrap™ Q column analysis

A HiTrap™ Q column was washed with 10 column washes of ultrapure water to remove the 20% ethanol used for storage of the column. The column was then equilibrated with 40 column washes of denaturing buffer. The sample was then passed through the column and the flow through was collected. 10 column washes of each of the following denaturing buffers with altered NaCl concentrations were then passed through: 10 mM NaCl, 50 mM NaCl, 150 mM NaCl and 500 mM NaCl. All washes were collected in 1.5 ml Eppendorf tubes and analysed by dot blotting for the presence of His tag.

2.4.4 HisTrap™ column analysis

Samples positive for His tag were then dialysed twice into 325 ml Nickel column buffer [4 x PBS, 8 M urea, 20 mM imidazole] for 3 hours each time using SnakeSkin™ Dialysis Tubing (Pierce). The column was washed with 10 column washes of ultrapure water to remove the 20% ethanol used for storage of the column and then equilibrated with 60 column washes of Nickel column buffer. The sample was then passed through the column and the flow through was collected. 10 column washes of Nickel column buffer was then passed through the column followed by 10 column washes of Elution buffer [4 x PBS, 8 M urea, 0.5 M imidazole].

2.4.5 Pulse Refolding

To refold protein after denaturing purification, the sample was dialysed in pulse refolding buffer [200 mM Tris.HCl (pH 8), 10 mM EDTA, 0.6 M arginine, 20% glycerol, 1 mM DTT, 50 mM NaCl] at a 1 in 100 dilution overnight at 4 ^{o}C. The sample was then dialysed into storage buffer [20 mM Tris.HCl (pH 8), 50 mM NaCl, 1 mM EDTA, 20% (v/v) glycerol, 1 mM DTT] twice for 3 hours each time at 4 ^{o}C using SnakeSkin™ Dialysis Tubing (Pierce).

2.4.6 Glycosylase Assay

Enzymatic activity of samples was monitored by apurinic site nicking produced by DNA glycosylase activity on double stranded oligonucleotide substrates as previously described (Sartori, 2001; Owen *et al.,* 2007).

The oligonucleotides employed were as follows and have a G:U mismatch (where F is fluoroscein):

5' – TAGACATTGCCCTCGAGGTACCATGGATCCGATGTCGACCTCAACCTAGACGAATTCCG – 3'
3' – ATCTGTAACGGGAGCTCCATGGTACCTAGGCTACAGUTGGAGTTTGGATCTGCTTAAGGC-F – 5'

The substrates were prepared by annealing oligonucleotides: a 0.5 M solution of the labelled lower strand and 1.0 M solution of the upper strand was heated at 95°C in 10 mM Tris HCl pH 8.0 (Sigma), 50 mM NaCl for 5 minutes. The solution was cooled to room temperature overnight. Assays were as follows: 50 nM labelled duplex DNA was incubated with 0.5 µl of sample in APE buffer (New England Biosciences), in the absence or presence of commercially available APE1 (New England Biosciences) for 60 minutes at 37°C.

Proteins were precipitated by adding 1/10 volume of 3 M sodium acetate pH 5.5 and then 3 volumes of 100 % ethanol. Samples were placed at –20°C overnight and then centrifuged at 14,000 rpm for 60 minutes. The supernatant was removed and the precipitates were rinsed with 70% ethanol (-20 °C) and air dried. Samples were re-suspended in loading buffer (15% TE pH 8.0, 85% deionised formamide), denatured at 95°C for 5 minutes, cooled on ice and resolved on a 15% (w/v) denaturing (7 M urea) polyacrylamide gel (acrylamide-bis ratio of 29:1, National Diagnostics). Reaction products were visualised using a Storm 840 phosphorimager using ImageQuant software.

2.5 Computational Methods

2.5.1 Densitometry Analysis using Scion Image

Image data from PCR analysis and glycosylase assays were analysed using Scion Image Software (http://www.scioncorp.com). Signal intensities were quantified by measuring the mean pixel intensity of bands of interest and values for PCR analysis were expressed as gene of interest over reference gene.

2.5.2 Analytical Digital Photomicroscopy

Image data obtained from immunohistochemistry were analysed using analytical digital photomicroscopy (ADP) as outlined by the Biocolor Company, UK. The digitally acquired images were posterized using Adobe® Photoshop® software where the blue pixels (as the substrate

conversion resulted in a blue stain) were counted in each image and recorded to give semi-quantitative values for comparison analysis. All photographs were taken at a constant magnification, all fields of view were of similar confluence and three photographs were taken of each slide to get an average pixel value.

The pathway utilised for the Adobe® Photoshop® software was:

Layer > New adjustment layer > Posterize (a consistent value of 4 utilised for all images) > Layer > Flatten image > Select > Color Range > Blues > OK > Histogram > Pixels

Chapter 3

Determination of an interaction between sMBD4 and MLH1

3 Determination of an interaction between sMBD4 and MLH1

3.1 Introduction

Approximately two-thirds of cases of colorectal cancer are due to a genetic predisposition where there are germ-line mutations in genes involved in the DNA mismatch repair system particularly MLH1 and MSH2 (Jacob and Praz, 2002). Therefore understanding the proteins involved in mismatch repair and their interactions with other proteins is key to understanding the pathogenesis of colorectal cancer. A protein demonstrating interaction with MLH1 via yeast two hybrid system has been identified and termed MBD4 (Bellacosa *et al.,* 1999; Macpartlin *et al.,* 2003). However, a more recent study aimed at identifying interacting partners of MLH1 using tandem affinity purification failed to show an interaction between MLH1 and MBD4 (Cannavo *et al.,* 2007) which highlights the question as to whether MLH1 and MBD4 physically interact with each other. To date the significance of any interaction between these two proteins has not fully been elucidated.

Recently a novel alternatively spliced form of MBD4 has been identified and termed sMBD4 (Owen *et al.,* 2007). Whilst retaining the glycosylase domain seen in MBD4, sMBD4 does not possess the canonical methyl-binding domain (Figure 3.1). Protein interactions of sMBD4 may be valuable for understanding of protein interaction of MBD4 as they share a common domain and so assist in elucidating the role of these proteins in DNA MMR. MBD4 and sMBD4 share a common glycosylase domain and this is the region that is said to bind to MLH1 (Kondo *et al.,* 2005). Essentially it may be determined if sMBD4 and MLH1 are binding partners and also the strength of association between MLH1 and the glycosylase domain of MBD4 can be ascertained.

3.2 Chapter Aims

The aims of this study were to establish a native protein purification method for sMBD4 and to identify whether sMBD4 and MLH1 were binding partners by demonstrating an interaction between the two proteins.

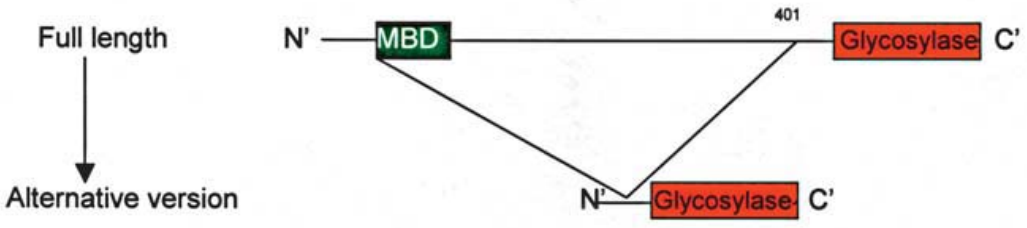
Figure 3.1 sMBD4 retains the glycosylase domain but does not have a Methyl-Binding Domain (MBD).

(a). Previously reported full length MBD4 sequence (Hendrich *et al.,* 1999). **(b).** Schematic diagram of MBD4 in comparison to predicted sMBD4 sequence. Splicing was seen to occur between the nucleotides coding for cysteine and arginine (codons82/83) and glutamate (codon 401) residues, and results in the conversion of glutamate 401 to a glutamine in sMBD4. 319 amino acids are deleted (underlined) to form sMBD4. **(c).** Predicted sMBD4 sequnce showing the potential nuclear localisation and the glycosylase domain. Putative nuclear localisation signals are indicated (yellow) (Bellacosa *et al.,* 1999). The MBD (green) is denoted here to span codons 82-135. The glycosylase domain is highlighted in red (Zhu *et al.,* 2000; Wu *et al.,* 2003). (Figure and Figure Legend taken with permission from Owen *et al.,* 2007).

(a)

1 MGTTGLESLS LGDRGAAPTV TSSERLVPDP PNDLRKEDVA MELERVGEDE
51 EQMMIKRSSE CNPLLQEPIA SAQFGATAGT EC*RKSVPCGW ERVVKQRLFG
101 KTAGRFDVYF ISPQGLKFRS KSSLANYLHK NGETSLKPED FDFTVLSKRG
151 IKSRYKDCSM AALTSHLQNQ SNNSNWNLRT RSKCKKDVFM PPSSSSELQE
201 SRGLSNFTST HLLLKEDEGV DDVNFRKVRK PKGKVTILKG IPIKKTKKGC
251 RKSCSGFVQS DSKRESVCNK ADAESEPVAQ KSQLDRTVCI SDAGACGETL
301 SVTSEENSLV KKKERSLSSG SNFCSEQKTS GIINKFCSAK DSEHNEKYED
351 TFLESEEIGT KVEVVERKEH LHTDILKRGS EMDNNCSPTR KDFTGEKIFQ
401 *EDTIPRTQIE RRKTSLYFSS KYNKEALSPP RRKAFKKWTP PRSPFNLVQE
451 TLFHDPWKLL IATIFLNRTS GKMAIPVLWK FLEKYPSAEV ARTADWRDVS
501 ELLKPLGLYD LRAKTIVKFS DEYLTKQWKY PIELHGIGKY GNDSYRIFCV
551 NEWKQVHPED HKLNKYHDWL WENHEKLSLS

(b)



(c)

1 MGTTGLESLS LGDRGAAPTV TSSERLVPDP PNDLRKEDVA MELERVGEDE
51 EQMMIKRSSE CNPLLQEPIA SAQFGATAGT EC* QDTIPRTQ IERRKTSLYF
101 SSKYNKEALS PPRRKAFKKW TPPRSPFNLV QETLFHDPWK LLIATIFLNR
151 TSGKMAIPVL WKFLEKYPSA EVARTADWRD VSELLKPLGL YDLRAKTIVK
201 FSDEYLTKQW KYPIELHGIG KYGNDSYRIF CVNEWKQVHP EDHKLNKYHD
251 WLWENHEKLS LS

3.3 Methods

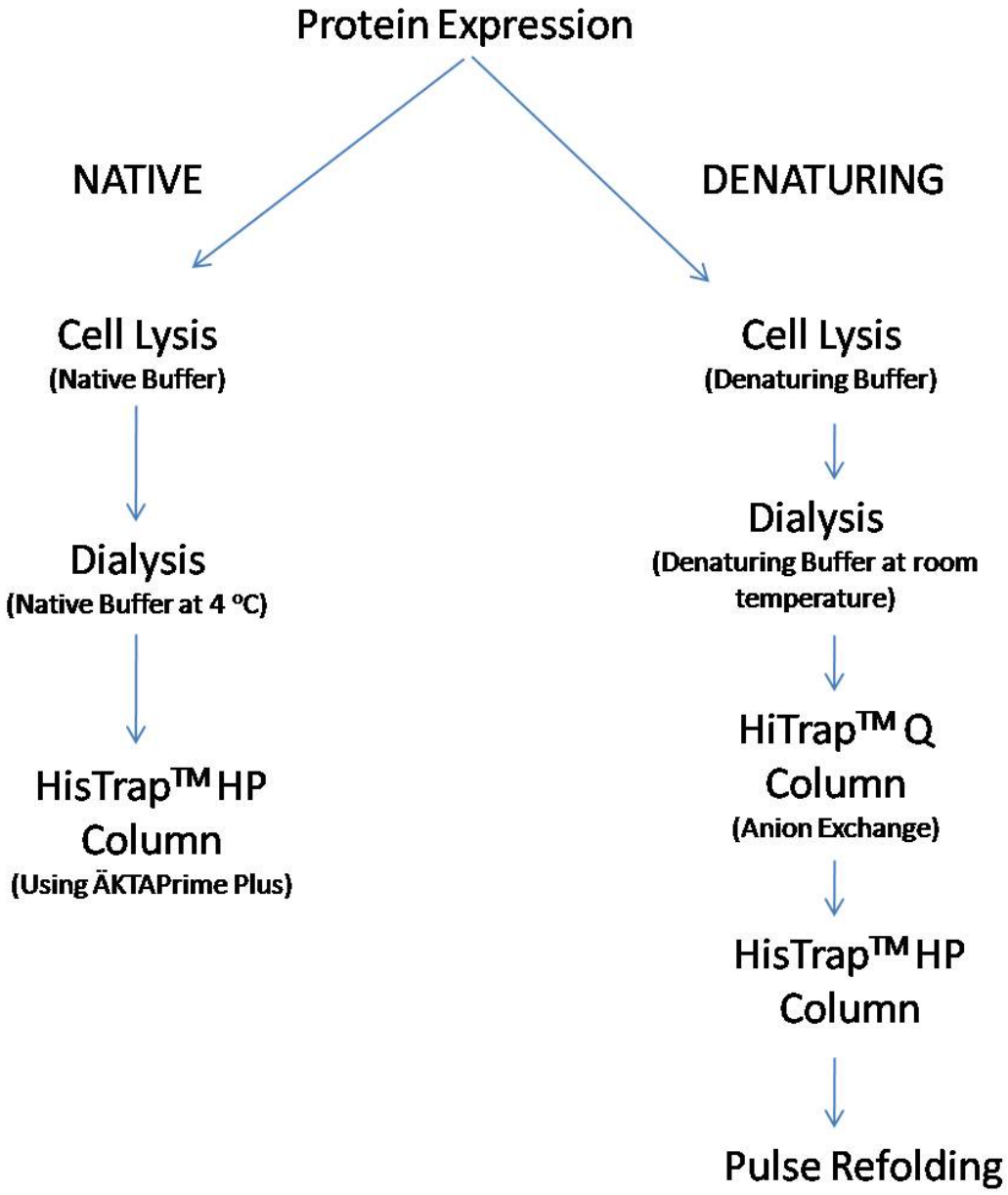
3.3.1 Protein Purification

For protein binding assays it was essential to have purified, functional sMBD4 and MLH1 protein. sMBD4 has been previously purified by a denaturing method of protein extraction followed by pulse refolding of the protein to recover activity (Owen *et al.,* 2007) (Chapter 2 'Materials and Methods', Sections 2.4.3 and 2.4.6) and is briefly summarised in Figure 3.2. This method extracted protein from *E. coli* under denaturing conditions. sMBD4 was then purified via anion exchange chromatography utilising a HiTrap™ Q column (GE HealthCare) and nickel affinity chromatography utilising a HisTrap™ HP column (GE HealthCare) which contains a Ni Sepharose matrix with a high affinity for histidine and therefore the His-tagged sMBD4. The purified protein was then pulse refolded against an arginine rich buffer.

This present study aimed to purify sMBD4 by a native purification technique whereby protein extracted from *E. coli* would be passed through a HisTrap™ HP column (GE HealthCare) and a gradient elution using Native elution buffer [2 x PBS, 500 mM NaCl, 500 mM imidazole] to elute the His-tagged sMBD4 utilising a pre-programmed application template setting for the ÄKTAPrime Plus specifically designed to purify His-tagged recombinant proteins. Essentially, recombinant sMBD4 would be purified in its native form from *E. coli* using this method and therefore eliminating the need to denature the protein, then utilise anion exchange chromatography and pulse refold to obtain functional purified protein. It was considered that this one-step method of purification would provide an easier and more economical way to yield a high amount of functional protein for use in protein binding assays (Figure 3.2 and described in detail in Chapter 2 'Materials and Methods', Section 2.4.2).

In addition, a commercially available recombinant GST-tagged MLH1 (Abnova, Taiwan) was utilised in this study.

Figure 3.2 Comparison of denaturing purification and native purification of sMBD4.

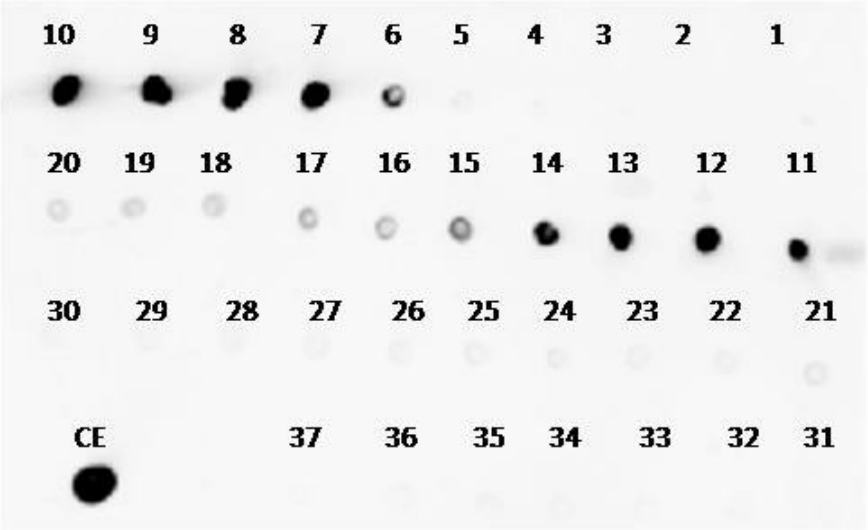


3.4 Purification of sMBD4 utilising native purification technique

For native purification of sMBD4, *E coli* expression vectors were obtained from Dr Iain Nicholl – University of Wolverhampton [sMBD4 cDNA cloned into pQE30 expression vector (Qiagen) using strain SG13009[pREP] (Qiagen) as a host] and cultures were grown (Chapter 2 'Materials and Methods', Section 2.4.1). (See Appendix for pQE30 map and sequence details).

Protein purification was performed using the ÄKTAPrime Plus system. Protein was extracted from *E. coli* under native conditions and was passed through a HisTrap™ HP column (GE Healthcare) that had been equilibrated with 2 x PBS, 500 mM NaCl, 20 mM imidazole. Protein was eluted with a linear imidazole gradient generated by mixing the aforementioned buffer with 2 x PBS, 500 mM NaCl and 500 mM imidazole. It was anticipated that contaminants would be removed by utilising a linear imidazole gradient which would elute a purified His-tagged protein. The eluted fractions were analysed by dot blotting for presence of His-tag (Figure 3.3) and were further analysed by immunoblotting (Figure 3.4) and silver staining (Figure 3.5) to determine purity of eluted protein. However, the results indicated that the purified protein was non-functional and in addition the presence of contaminants was also detected. Removal of these contaminants was attempted by denaturating the purified protein sample and passing the sample over a HiTrap Q column (GE Healthcare) and then using a stepwise NaCl gradient to elute the proteins. The sample was then passed again over the HisTrap™ HP column using a stepwise imidazole gradient, to attempt to remove any contaminants present in the sample. However, this method was unsuccessful in purifying a high yield of protein necessary for protein-protein interaction assays and was also unsuccessful in eliminating contaminants in the protein sample.

Figure 3.3. Dot blot analysis of chromatographic fractions collected from the ÄKTAprime plus. The thirty seven 1 ml fractions collected from the ÄKTAprime plus were analysed for presence of His-tag by dot blot analysis using anti-His HRP conjugated antibody (Sigma). 0.5µl of each fraction was dotted onto the nitrocellulose membrane. CE = Crude Extract.



The dot blot showed that fractions 7 to 14 were positive for His-tag and therefore indicated presence of sMBD4 being eluted in these fractions. The protein was precipitated from these fractions according to Wessel and Flugge (1984) as described in Chapter 2 'Materials and Methods', Section 2.3.3 and then analysed by immunoblotting (Figure 3.4) and silver staining (Figure 3.5) to determine the molecular mass and purity of protein in the fractions.

Figure 3.4. Immunoblot analysis of His-tag positive fractions collected from the ÄKTAprime plus. Immunoblot analysis was carried out as described in 'Materials and Methods' (Chapter 2, Section 2.3.5) to determine presence of His-tag using anti-His HRP conjugated antibody (Sigma). The results indicated a band at approximately 37 kDa in samples 9, 10 and 11 which corresponded to the molecular weight of His-tagged sMBD4. Number of lane corresponds to chromatographic fraction analysed.

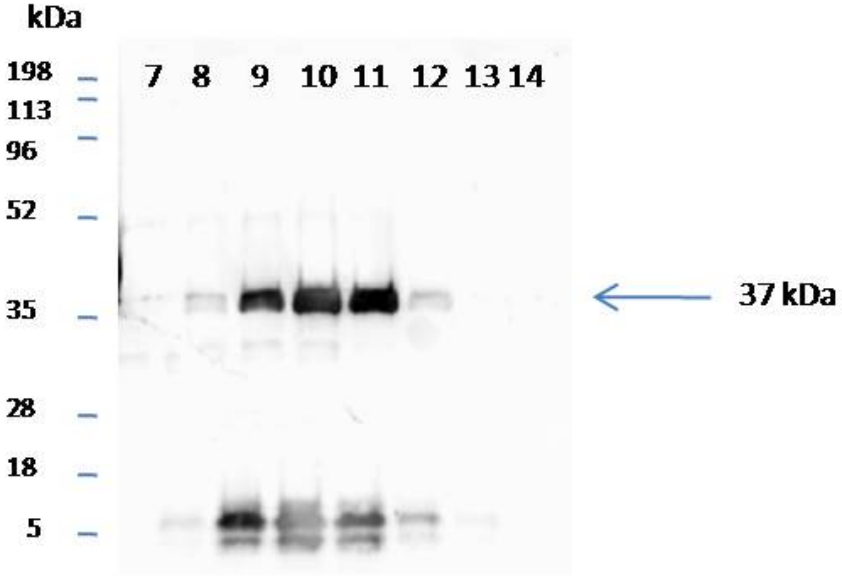
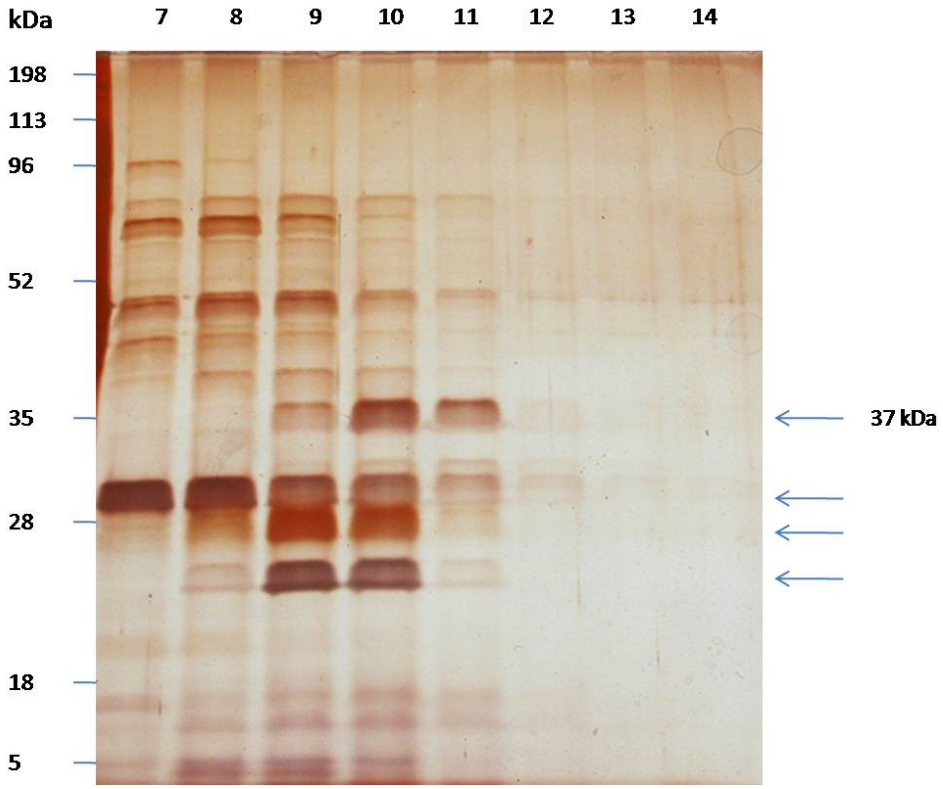
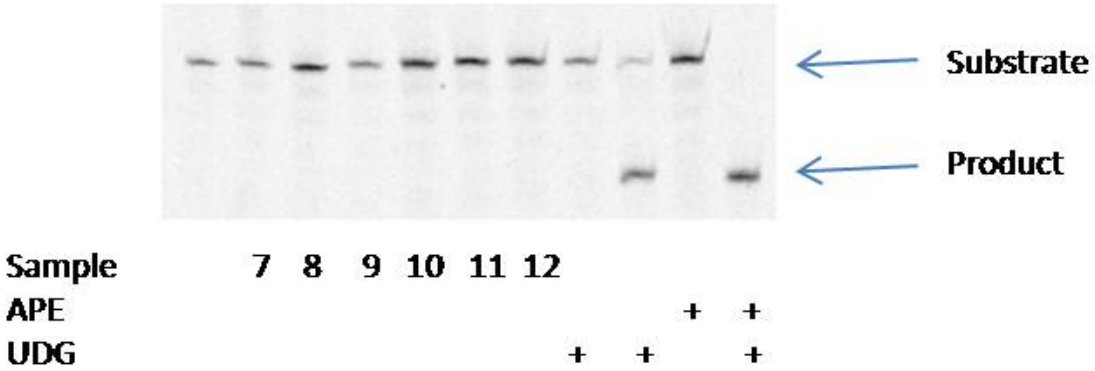


Figure 3.5. Silver Stain analysis of His-tag positive samples collected from the ÄKTAprime plus. Silver staining was carried out as described in 'Materials and Methods' (Chapter 2, Section 2.3.6.2). As with immunoblot analysis a molecular band at approximately 37 kDa which corresponded to the size of His-tagged sMBD4 was observed**.** However, three major bands, smaller in size (indicated by arrows), were also observed. Lane numbers correspond to chromatographic fractions analysed.



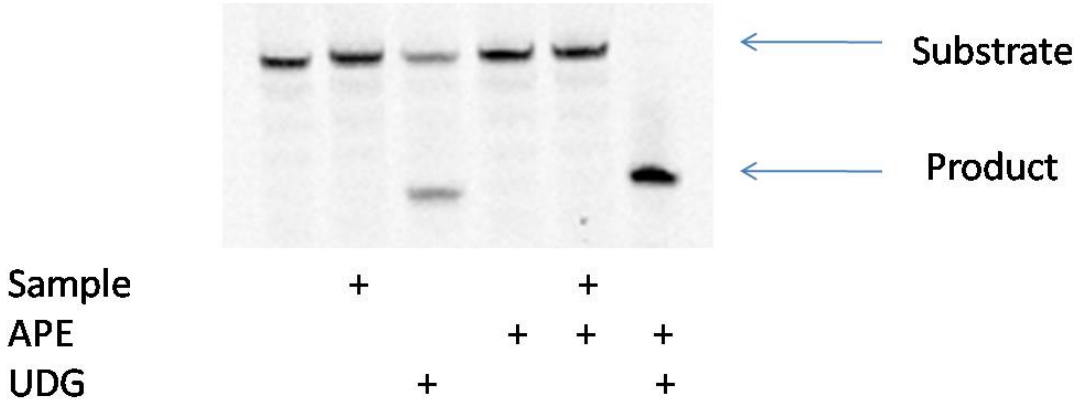
Immunoblotting indicated a strong band at approximately 37 kDa (Figure 3.4) particularly in samples 9, 10 and 11 suggesting successful purification of sMBD4. However, further analysis by silver staining (Figure 3.5) showed the presence of 3 major bands smaller in size. As these bands were not detected by immunoblotting it is most likely that these bands represented contaminants rather than breakdown products. However, although there was evidence of contaminants present in the samples, they indicated that sMBD4 had been successfully purified via this native purification method so the samples were assayed for presence of functional sMBD4 protein utilising a glycosylase assay as described in Chapter 2 'Materials and Methods', Section 2.4.7. The samples collected from the ÄKTAprime plus which were positive for His-tag and indicated a band at the correct molecular weight for sMBD4 were analysed for repair activity (Figure 3.6) utilising a repair assay. Briefly, this technique employs double stranded oligonucleotide substrate with a G:U mismatch and a fluoresein molecule. The substrates were taken and incubated with sMBD4, another DNA glycosylase (UDG) (as a positive control) and/or AP Endonuclease 1 (APE1). sMBD4 as a DNA glycosylase recognises the G:U mismatch and the APE1 nicks the DNA resulting in two halves. The samples were run on a denaturing polyacrylamide gel and then analysed using a fluorescence scan for presence of the fluorescein molecule which indicated DNA glycosylase activity. Results however, failed to show evidence of repair activity in any of the samples suggesting that the protein purified in the samples was non-functional, if it indeed was sMBD4 (Figure 3.6).

Figure 3.6. Glycosylase Assay analysis of protein samples positive for His-tag collected from the ÄKTAprime plus. The protein samples showed no evidence of glycosylase activity. Sample number corresponds to chromatographic fraction analysed. (APE = AP Endonuclease; UDG = Uracil DNA glycosylase).



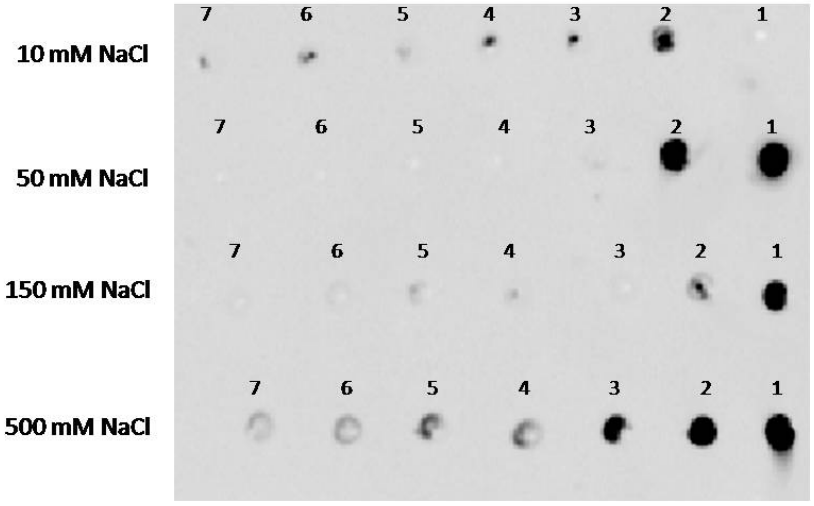
The protein samples were pooled and dialysed into Storage Buffer [20 mM Tris HCl (pH 8), 50 mM NaCl, 1 mM EDTA, 20% (v/v) glycerol, 1 mM DTT] twice for 3 hours each time at 4 ^{o}C using SnakeSkin^{TM} Dialysis Tubing (Pierce) to remove imidazole from the native elution buffer (as a possible inhibitor of the glycosylase activity) and then again assayed for presence of functional protein. Results failed to show any evidence of repair activity indicating non-functional protein (Figure 3.7).

Figure 3.7. Glycosylase Assay with protein sample dialysed in storage buffer [20 mM Tris HCl (pH 8), 50 mM NaCl, 1 mM EDTA, 20% (v/v) glycerol, 1 mM DTT]. The protein sample showed no evidence of glycoylase activity. (APE = AP Endonuclease; UDG = Uracil DNA Glycosylase)



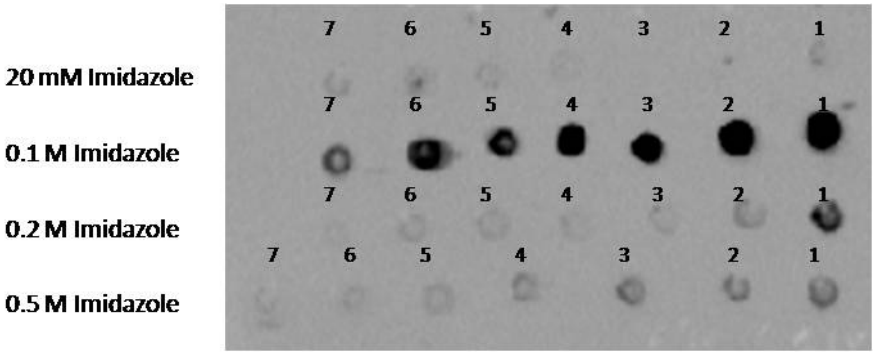
In an attempt to recover the protein and remove the contaminants seen by silver staining, the samples were denatured by dialysing against Denaturing Buffer [25 mM Tris HCl pH 8 (Sigma), 8 M urea (Sigma), 10 mM NaCl, 1 mM EDTA, 1 mM DTT] for 3 hours twice. To remove contaminating proteins the sample was passed over a HiTrap Q column and step elution was carried out using denaturing buffers with altered NaCl concentrations (50 mM, 150 mM and 500 mM). The eluted fractions were then analysed for presence of His-tag by dot blotting. Results indicated His-tag presence in 50 mM NaCl samples and 500 mM NaCl samples (Figure 3.8).

Figure 3.8. Dot Blot analysis of fractions collected from denatured sample step elution over HiTrap Q Column. The fractions were analysed for presence of His-tag by dot blot analysis using anti-His HRP conjugated antibody (Sigma). Evidence of His-tag in 50 mM NaCl elution and 500 mM NaCl elution fractions. Numbers represent fractions collected for each NaCl concentration.



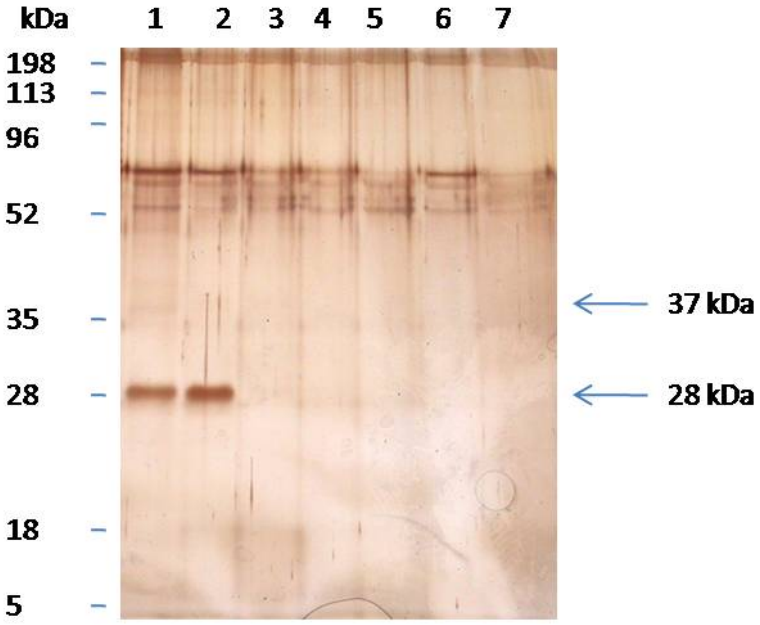
The 50 mM NaCl samples were pooled as it was expected that these samples would have fewer contaminants than 500 mM NaCl samples and dialysed into HisTrap™ HP column buffer [4 x PBS (Sigma), 20 mM Imidazole (Sigma), 8 M urea (Sigma)] for 3 hours twice. The sample was then passed over the HisTrap™ HP column and proteins were eluted with a step-wise gradient using buffers of increasing imidazole concentration (0.1 M, 0.2 M, 0.5 M). The eluted fractions collected were again analysed for presence of His-tag and results indicated that the His-tag was present in samples eluted using 0.1 M imidazole concentration (Figure 3.9).

Figure 3.9. Dot blot analysis of fractions eluted from HisTrap™ HP column using a step wise imidazole gradient. The fractions were analysed for presence of His-tag by dot blot analysis using anti-His HRP conjugated antibody (Sigma). Evidence of His-tag elution in 0.1 M imadazole fractions. Numbers represent fractions collected for each imidazole concentration.



Protein was precipitated from the fractions collected from the 0.1 M imidazole elution and analysed by silver stain for analysis of molecular weight and purity of the protein purified. However silver stain analysis indicated sample degradation and a contaminant at approximately 28 kDa was also eluted (Figure 3.10). It was therefore decided not to refold the protein and analyse for repair activity as there was very little protein present. Also, the contaminant being close in size to sMBD4 would be difficult to separate by size and therefore the purity of sMBD4 purified by any further subsequent steps would be compromised.

Figure 3.10. Silver Stain analysis of 0.1 M imidazole fractions eluted from HisTrap™ HP column. Very little protein at approximate size for sMBD4 (37 kDa) and evidence of a contaminant at approximately 28 kDa can be observed (as indicated by arrow). Numbers represent fractions collected for 0.1 M imidazole elution.



3.5 Determination of an interaction between sMBD4 and MLH1

3.5.1 Analysis of recombinant proteins utilised for determination of interaction.

For this present study, commercially available recombinant GST-tagged MLH1 (Abnova, Taiwan) was utilised. Immunoblot analysis (Figure 3.11A) and silver stain analysis (Figure 3.11B) was carried out to confirm the molecular weight and purity of the protein received from the company. Due to unsuccessful attempts to purify sMBD4 utilising a native purification technique (as described in Section 3.3), sMBD4 was purified by the denaturing purification technique (Owen *et al.,* 2007). Purified protein was analysed by immunoblotting (Figure 3.12A) and assayed for glycosylase activity (Figure 3.12B) for evidence of functional protein.

Figure 3.11. Analysis of commercially available MLH1 (Abnova, Taiwan). Protein was precipitated using Wessel and Flugge method as described in Chapter 2 'Materials and Methods', Section 2.3.3 and then subject to SDS-PAGE analysis **(A)** Sample was analysed for presence of GST-tag by immunoblot analysis using anti-GST HRP conjugated antibody (Sigma). Analysis shows a strong band at approximately 109 kDa suggesting presence of GST-tagged MLH1. However, there are further bands below suggesting presence of breakdown products **(B)** Sample was analysed by Silver Staining to determine purity of sample. Analysis demonstrates presence of a strong band at approximately 109 kDa and also presence of a break down product at approximately 27 kDa (corresponds to approximate size of GST tag).

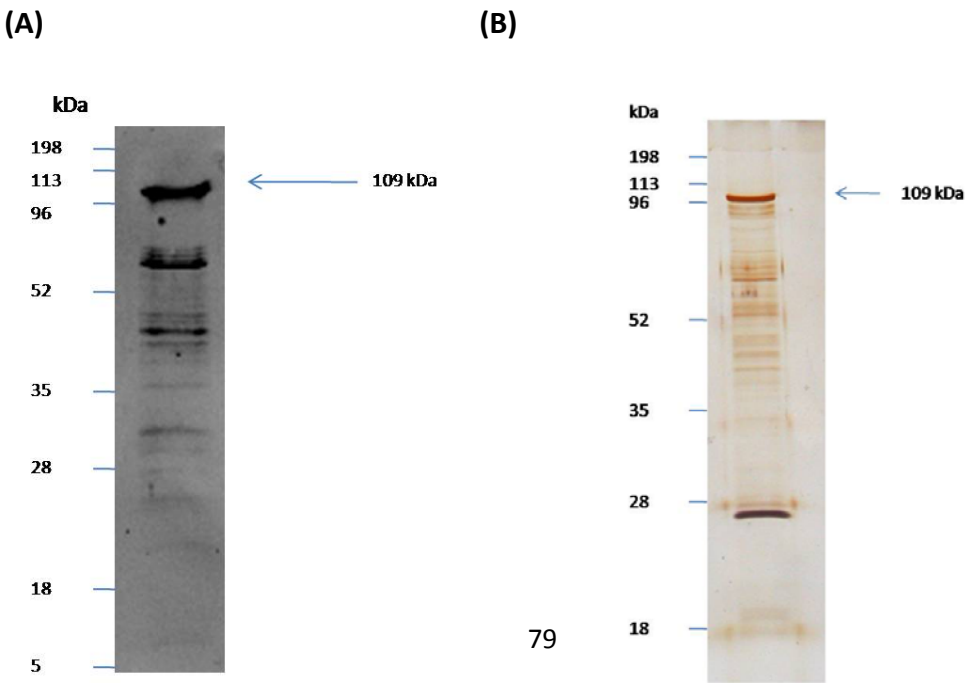
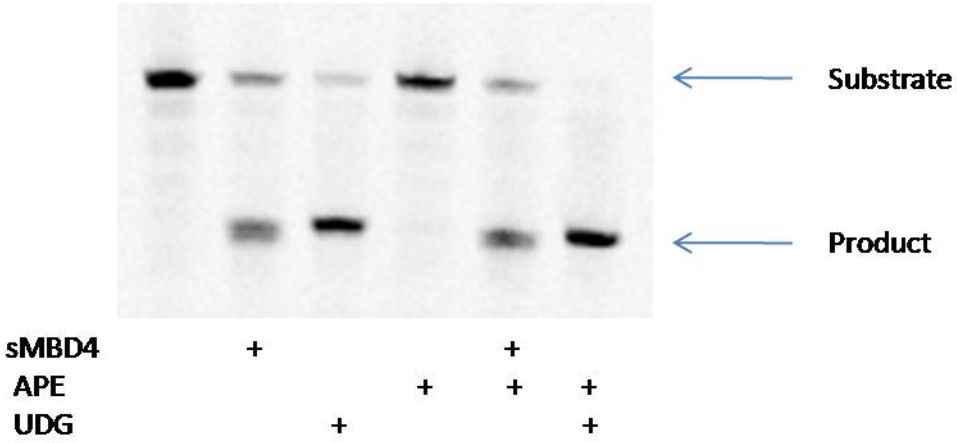


Figure 3.12 Glycosylase assay to demonstrate purified sMBD4 functional activity. (APE = AP Endonuclease; UDG = Uracil DNA Glycosylase)

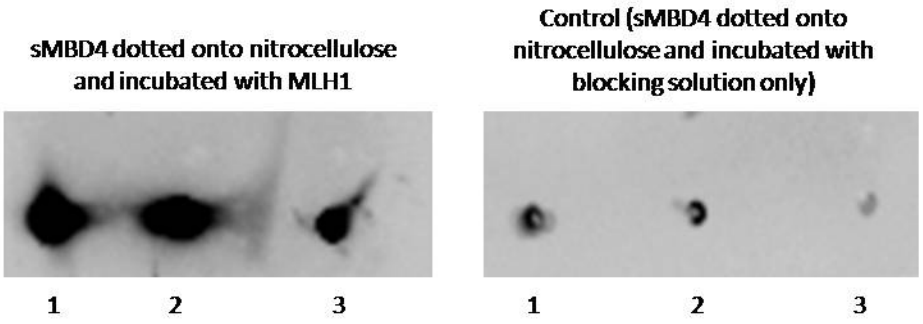


3.5.2 Dot Blotting Binding Assays

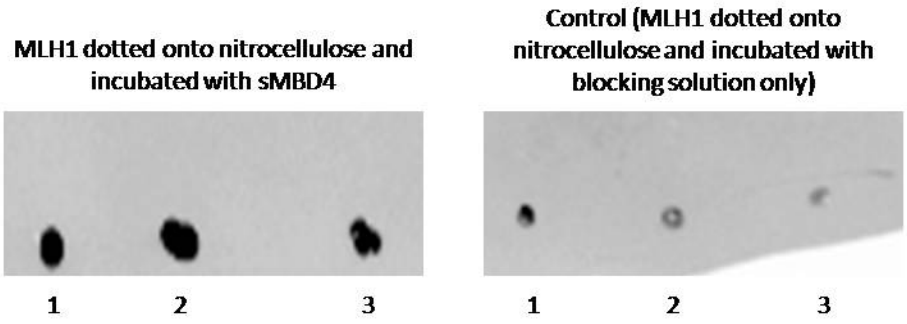
Using the recombinant proteins simple binding assays were initially tried. Briefly, MLH1 was dotted directly onto nitrocellulose membrane, blocked to prevent non-specific binding, then incubated with sMBD4, washed and then analysed for presence of sMBD4 using His-tag specific antibody (Figure 3.13A). The converse experiment was also carried out with sMBD4 dotted directly onto nitrocellulose membrane, blocked to prevent non-specific binding, then incubated with MLH1, washed and then analysed for presence of MLH1 using GST-tag specific antibody (Figure 3.13B). Although initial results suggested an interaction between the two proteins (Figure 3.13A and Figure 3.13B), after several further attempts it was found that the results were not reproducible (data not shown).

Figure 3.13 Dot blotting binding assays to determine an interaction between MLH1 and sMBD4. **(A)** Detection of MLH1 binding to sMBD4. **(B)** Detection of sMBD4 binding to MLH1. 1 = 1 µl, 2 = 0.5 µl, 3 = 0.1 µl of protein sample dotted onto membrane

(A)



(B)

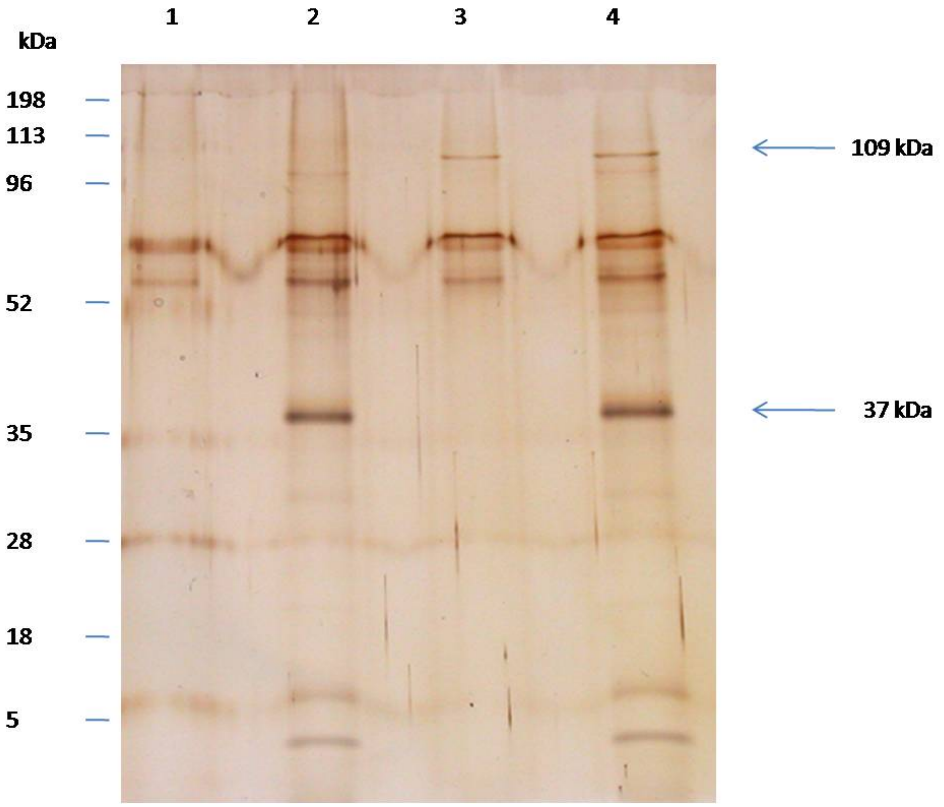


3.5.3 Immunoprecipitations

An immunoprecipitation technique was also attempted to determine a specific protein-protein interaction between sMBD4 and MLH1. EZview™ Red HIS-select™ HC Nickel Affinity gel was utilised in this study. This gel contains beads which have a high affinity for histidine and therefore the recombinant His-tagged sMBD4. sMBD4 and MLH1 were incubated with the beads and it was expected that sMBD4 and MLH1 would bind to form a complex and that this protein complex would then bind to the beads via the histidine tag on the sMBD4. Subsequently, protein bound to the beads could then be eluted off and analysed by SDS-PAGE and any protein-protein interaction could be determined. However, as Figure 3.14 shows the recombinant GST-tagged MLH1 used in the study showed some affinity to the beads (Lane 3).

Figure 3.14 Silver stain analysis of immunoprecipiatation of sMBD4 and MLH1 utilising EZview™ Red HIS-select™ HC Nickel Affinity gel.

Lane 1 = Bead alone, Lane 2 = Beads and sMBD4, Lane 3 = Beads and MLH1, Lane 4 = Beads, sMBD4 and MLH1

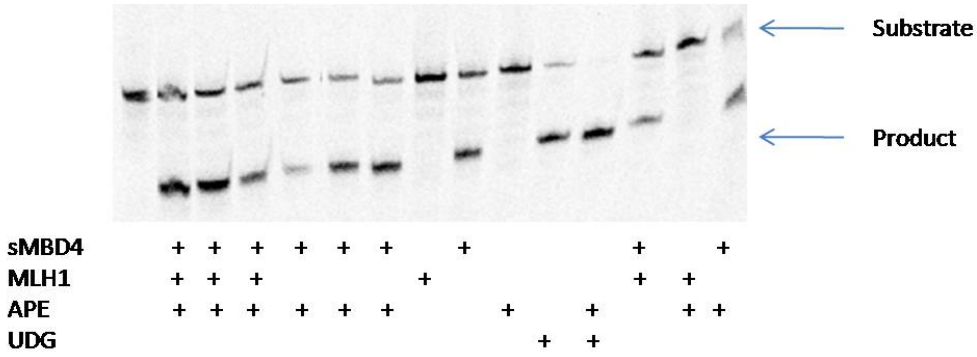


3.5.4 Glycosylase assay activity

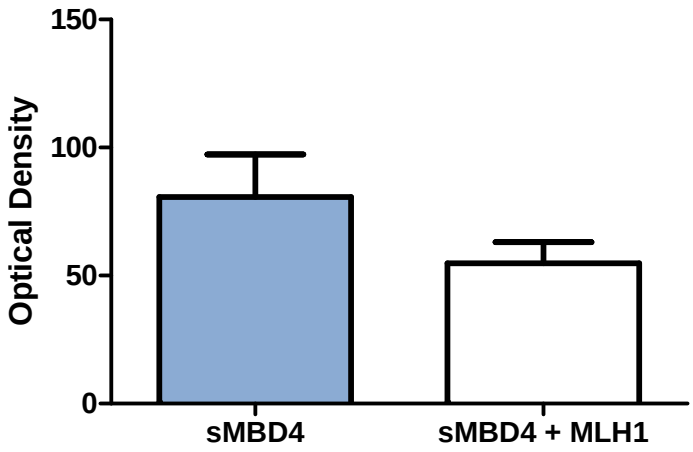
sMBD4 has glycosylase activity (Owen *et al.,* 2007) and it was hypothesised that if sMBD4 and MLH1 interact it may affect sMBD4 glycosylase activity. To test this hypothesis a glycosylase assay was employed (as described in Chapter 2 'Materials and Methods', Section 2.4.6). The assay was repeated with addition of MLH1 to sMBD4 to investigate its effects on sMBD4 activity (Figure 3.15A). Analysis of band density of the product band showed a slight non - significant decrease in product when sMBD4 is incubated with MLH1 suggesting sMBD4 glycosylase activity was affected by MLH1 addition (Figure 3.15B).

Figure 3.15 Glycosylase assay to determine if MLH1 affects sMBD4 glycosylase activity. **(A)** Glycosylase assay. **(B)** The optical density of the bands visualised by gel electrophoresis was measured by Scion Image analysis. The graph display the optical density measured for product bands of sMBD4 activity alone and sMBD4 activity when incubated with MLH1. Significant difference in optical density was measured using an unpaired t-test. (P = 0.2337). n = 3 (APE = AP Endonuclease; UDG = Uracil DNA Gylcosylase)

(A)



(B)



3.6 Discussion

3.6.1 Purification of Protein

The aim of this present study was to demonstrate an interaction between MLH1 and sMBD4. It was hoped that further investigations could then be carried out into identifying the specific binding domain and whether mutations in the binding site and subsequent lack of protein binding played a role in the pathogenesis of colorectal cancer. Essential to this study was purified, functional protein and therefore initially, attempts were made to purify sMBD4 using a novel native purification technique as an alternative method to the Owen *et al.,* (2007) denaturing purification method in hope of obtaining higher purity sMBD4 and attempt to maintain protein stability and activity by eliminating the denaturation of the protein. However, degradation of sMBD4 was greatly increased and protein purified via this method failed to show any repair activity demonstrating that the protein purified was non-functional which negated the benefits of this purification technique. Therefore, denaturing extraction and refolding of sMBD4 may prove to be a more efficient method for protein recovery and retention of protein activity.

3.6.2 Protein Interaction Assays

Utilising sMBD4 purified as described by Owen *et al.,* (2007) and a commercially available recombinant MLH1 (Abnova, Taiwan) assays were carried out to determine a specific protein-protein interaction between the two proteins. Initially simple dot blot binding assays using the recombinant proteins were attempted and results indicated sMBD4 and MLH1 binding (Figure 3.13A and Figure 3.13B). However, after several further attempts it was found that the results were not reproducible. This suggested that either the binding was non-specific, or possibly that the immobilisation of proteins onto a membrane may result in variations of orientation (Neves-Petersen *et al.,* 2006). Binding domains may then vary every time making determination of a specific protein-protein interaction via this method unreliable. It may also be that the reagents used to immobilise the protein may affect the protein structure and/or function which may subsequently affect protein interaction (Neves-Petersen *et al.,* 2006).

Immunoprecipitation technique utilising EZview™ Red HIS-select™ HC Nickel Affinity gel which have a high affinity for histidine and therefore the recombinant sMBD4 which had a histidine tag was also attempted to determine a specific protein-protein interaction. However, results showed

that the GST-tagged MLH1 bound non-specifically to the beads (Figure 3.14). Attempts were made to decrease non-specific binding by using different blocking solutions, however these proved to be unsuccessful. After several attempts and method modification, results continued to show that there was a non-specific interaction between MLH1 and the His-select beads making a specific protein-protein interaction between sMBD4 and MLH1 difficult to elucidate via this method.

3.6.3 Glycosylase Assay

Glycosylase assays were attempted to determine if MLH1 interacted with sMBD4 and affected glycosylase activity. This method is based on the glycosylase activity of sMBD4. Briefly, this technique employs double stranded oligonucleotide substrate with a G:U mismatch and a fluoresein molecule. The substrate is incubated with sMBD4 and APE1 (a DNA glycosylase). sMBD4 recognises the G:U mismatch and APE1 nicks the DNA resulting in two halves. The samples were then run on a denaturing polyacrylamide gel and then analysed using a fluorescence scan for presence of the fluoresein molecule. This experiment was repeated with addition of MLH1 to investigate its effects on sMBD4 activity. Interestingly, analysis of band density of the product bands from the glycosylase assay showed a slight although non significant decrease in band density when sMBD4 was incubated with MLH1 suggesting that glycosylase activity of sMBD4 may be affected by the presence of MLH1 and the possibility that they may therefore interact and that this interaction may affect sMBD4 glycosylase activity. MBD4 is reported to interact with MLH1 via the glycosylase domain (Kondo *et al.,* 2005) therefore it is possible that any interaction between sMBD4 and MLH1 will affect sMBD4 glycosylase activity.

It may be that a specific protein-protein interaction was not observed in this study as a 'third party' molecule may be required for binding of these proteins to occur *in vivo* or it may be that a specific event such as DNA damage needs to occur which may trigger a cascade which results in these two proteins binding. It may also be that environmental factors such as specific salt concentration are required and these need to be considered as reasons as to why a specific protein-protein interaction could not be determined via the methods used in this present study. Although this study utilised dot blotting, immunoprecipitations and glycosylase activity assays to ascertain a protein-protein interaction between sMBD4 and MLH1, there are other methods which may be employed to further investigate for example BiaCore analysis. Biacore utilises

surface plasmon resonance (SPR) technology to determine specificity of interaction between molecules and also the kinetics and affinity can also be characterised (GE Healthcare).

Although the results presented in this chapter fail to show a direct protein-protein interaction between sMBD4 and MLH1, this does not mean that such an interaction does not occur. Ruzov *et al.,* (2009) have recently shown co-localisation of recombinant MLH1 and MBD4 specifically within the nucleus of HeLa cells and suggest that MBD4 recruits MLH1 to sites of methylated DNA. They also suggested that MLH1 and MBD4 are co-localised at sites of DNA damage. In addition they showed an interaction between mouse MLH1 and recombinant *Xenopus* MBD4 via pull-down assays. They support previous studies (Kondo *et al.,* 2005) which suggest that MBD4 interacts with MLH1 via its C-terminal glycosylase domain. Although this present study also utilised a co-immunoprecipitation technique, unlike the findings of Ruzov *et al.,* (2009) a specific interaction was not observed. This may be due to the different experimental conditions utilised which may have affected protein interaction.

However, it is interesting to observe in the literature that within the 10 years since MBD4 and MLH1 transcriptional coupling was initial reported in 1999 (Bellacosa *et al.,* 1999), to date very little data has been reported in support of this. It is possible to suggest that this may be due to other researchers observing a lack of interaction between the two proteins, as reported by Cannavo *et al.,* (2007), but have not reported the findings.

3.6.4 Conclusion

In this present study, native protein purification was not successful in obtaining purified, functional sMBD4. In addition a reliable demonstration of protein-protein interaction between sMBD4 and MLH1 could not be shown. Hence this aspect of the work was abandoned.

Chapter 4

Investigation into the Effects of Aspirin on the Transcription of DNA Repair and Related Signalling Pathway Genes

4 Investigation into the effects of aspirin on the transcription of DNA repair and related signalling pathway genes

4.1 Introduction

There is abundant evidence that regular ingestion of non-steroidal anti-inflammatory drugs (NSAIDS) such as aspirin can promote colorectal tumour regression and reduce the relative risk of developing colorectal cancer (Rosenberg *et al.,* 1991; Thun *et al.,* 1991; Muscat *et al.,* 1994; Samson *et al.,* 2001; Thun *et al.,* 2002; Baron *et al.,* 2003; Imperiale, 2003; Sandler *et al.,* 2003). There is also substantive evidence that aspirin is specifically cytotoxic against colorectal cancer cells cultured *in vitro* (Smith *et al.,* 2000; Stark *et al.,* 2001; Yin *et al.,* 2006; Luciani *et al.,* 2007). The molecular basis for this cytotoxicity is controversial, and as previously discussed (Chapter 1) there are a number of hypotheses. Of key interest to this study is the suggestion that aspirin induces expression of DNA mismatch repair (MMR) proteins, specifically MLH1 (Goel *et al.,* 2003).

Although the results presented in Chapter 3 indicated a lack of interaction between MLH1 and sMBD4, as discussed previously MLH1 and MBD4 have been shown to be transcriptionally coupled and have been reported to be binding partners (Bellacosa *et al.,* 1999, Macpartlin *et al.,* 2003). Given this research evidence, it was hypothesised if aspirin induces expression of MLH1 we may see a concomitant increase in MBD4 expression upon aspirin treatment. In addition, unpublished data (Dr Iain Nicholl: personal communication) showed preliminary evidence of an increase of *MBD4* gene expression in SW480 cells upon salicylic acid treatment by semi-quantitative PCR analysis. Thus, the primary aim of this investigation was to determine whether aspirin exposure affected MBD4 gene expression in colorectal cancer cells.

Further to this, the suggestion by Goel *et al.* (2003) that MMR proteins may be involved in the mechanism of action of aspirin is of interest and therefore one objective of this investigation was to determine whether aspirin affected expression of genes involved in DNA damage signalling pathways. This was done by two approaches – semi-quantitative and quantitative real-time PCR and PCR array. A commercially available PCR array was utilised to assess the expression of a panel of different genes involved in DNA damage signalling pathways upon aspirin treatment. Genes featured on the Human DNA Damage Signalling Pathways RT^2 Profiler™ array (SuperArray

Biosciences Corporation, Frederick, MD) included those associated with the ATR/ATM signalling network and transcriptional targets of DNA damage responses.

The human colorectal cancer cell line SW480 was chosen as the model for this study as it is MMR proficient (Liu *et al.,* 2002; Carethers *et al.,* 1996). The cell line was derived from a 50 year old patient and is a grade 4 Duke's Class B adenocarcinoma (Melcher *et al.,* 2000 ; Leibovitz *et al.,* 1976). The cell line has a p53 point mutation (Tomita *et al.,* 1992; Roschke *et al.,* 2003; Leibovitz *et al.,* 1976; Duranton *et al.,* 2003) but does have full length MLH1 and MSH2 (Carethers *et al.,* 1996). SW480 is a COX-2 negative cell line but does express low levels of COX-1 (Richter *et al.,* 2001).

4.2 Chapter Aims

In this study it was initially aimed to investigate the effects of aspirin exposure on MBD4 gene expression in SW480 cells by semi-quantitative PCR analysis and the more sensitive quantitative real-time PCR method.

Further to this a novel RT^2 Profiler™ PCR Array analysis was carried out to determine the effects of aspirin on expression of key genes involved in DNA damage signalling pathways in SW480 cells.

4.3 Analysis of the affects of aspirin and salicylic acid on cell viability of cancer cell lines

Preliminary studies were carried out to determine the cytotoxic effects of aspirin and salicylic acid on SW480 cell viability utilising the MTT cell viability assay. SW480 cells were incubated with aspirin or salicylic acid at a range of 0 – 10 mM in L-15 medium supplemented with 1% L-glutamine/penicillin/streptomycin and 10% FCS for 24, 48 or 72 hours as described in 'Materials and Methods' (Chapter 2, Section 2.1.5).

As noted by a number of other researchers (Smith *et al.,* 2000; Yin *et al.,* 2006; Stark *et al.,* 2001; Luciani *et al.,* 2007) it is noted that aspirin and also salicylic acid exhibits a dose-dependent reduction of cell viability of colorectal cell line SW480 (Figures 4.1 – 4.3) and also the colorectal cancer cell line HCT116 (Figures 4.7 – 4.9). This present study also shows that low levels of aspirin and salicylic acid are more toxic to colorectal cancer cells (SW480 and HCT116) than other cancer types including breast cancer (MCF7 and MDA231-MB) and glioma (U373MG) cell lines (Figures 4.7 – 4.9).

It was of particular interest to study the effect of aspirin at relatively low levels (approximately 1 mM) in comparison to other studies where microarray analysis has been utilised (Iizaka *et al.,* 2002; Germann *et al.,* 2003; Hardwick *et al.,* 2004; 2003; Yin *et al.,* 2006), to ensure that the results obtained were as physiologically relevant as possible. Salicylate levels of 1 mM have been reported to be achievable in serum in human subjects given analgesic doses of aspirin (Stark *et al.,* 2001; Dihlmann *et al.,* 2001; Stark *et al.,* 2007).

In addition, cell viability assays were also carried out to determine an appropriate diluent for aspirin and salicylic acid as various different diluents have been used in similar experimental research in previous studies. For example Hardwick *et al.,* (2004) utilised DMSO, Goel *et al.,* (2003) utilised Tris-HCl buffer and Stark *et al.,* (2001) utilised solubilised aspirin in water using 10 N NaOH to pH 7. The effects of the diluents 100% ethanol and Tris.HCL buffer (pH 7.2) on cell viability were analysed in this present study.

4.3.1 Aspirin affects SW480 cell viability

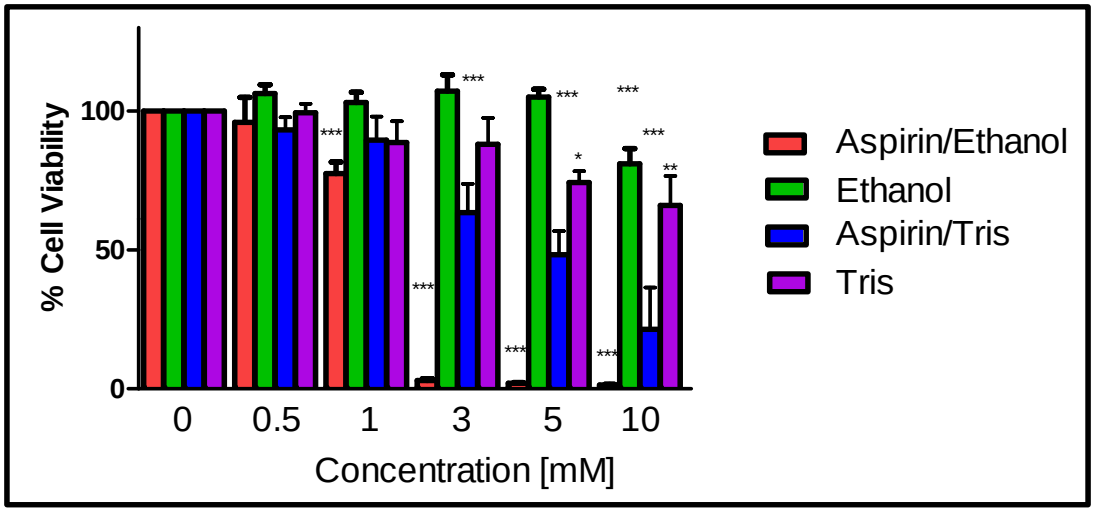
Hardwick *et al.,* (2004) and also Yin *et al.,* (2006) have shown that aspirin gives different gene expression patterns at different concentrations. Both studies show that at low concentrations (1 mM) aspirin affected a different set of genes in the colorectal cancer cell line HT-29 compared to when treated with 5 mM aspirin. For gene expression analysis it was therefore essential that we chose an appropriate concentration of aspirin at physiologically relevant low concentrations.

SW480 cell viability was analysed over a range of concentrations with particular attention paid to low physiologically relevant concentrations (0.5 mM and 1 mM) to see whether in our experimental model these low concentrations were indeed having an effect on cell viability. Changes in SW480 cell viability were monitored over 24, 48 and 72 hour time periods following treatment with increasing concentrations of aspirin (0.5 – 10 mM) (Figures 4.1 – 4.3). Aspirin demonstrated a concentration-dependent decrease in SW480 cell viability and also a time-dependent decrease as indicated in Figures 4.1 – 4.3.

Interestingly, the results showed that the effects of aspirin on cell viability may be dependent on the diluent used. After 24 hours, aspirin dissolved in ethanol caused almost total cell death after 24 hours at high concentrations i.e., 3 mM, 5 mM and 10 mM (Figure 4.1) with total cell death observed at these concentrations at 48 hours onwards (Figures 4.2 and 4.3). However, aspirin dissolved in Tris-HCl buffer (pH 7.2) did not cause total cell death even at the high concentration of 10 mM until 72 hours (Figure 4.3).

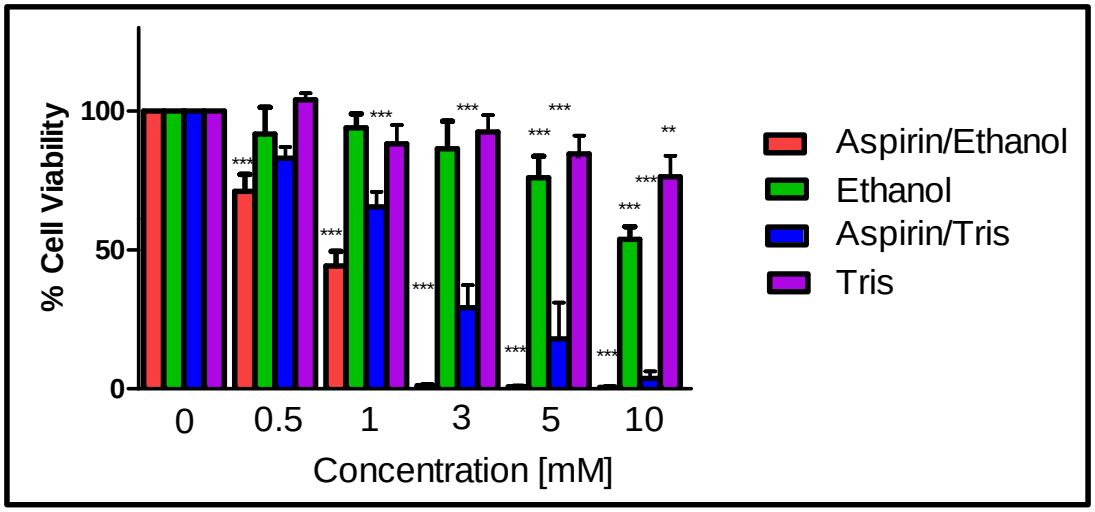
It is also notable that both diluents, 100% ethanol and Tris-HCl buffer (pH 7.2), particularly at high concentrations (5 mM and 10 mM), caused significant decreases in cell viability with 100% ethanol having a greater effect than Tris.HCl buffer (Figures 4.1 – 4.3).

Figure 4.1. Cell viability of SW480 cells treated with aspirin for 24 hours.



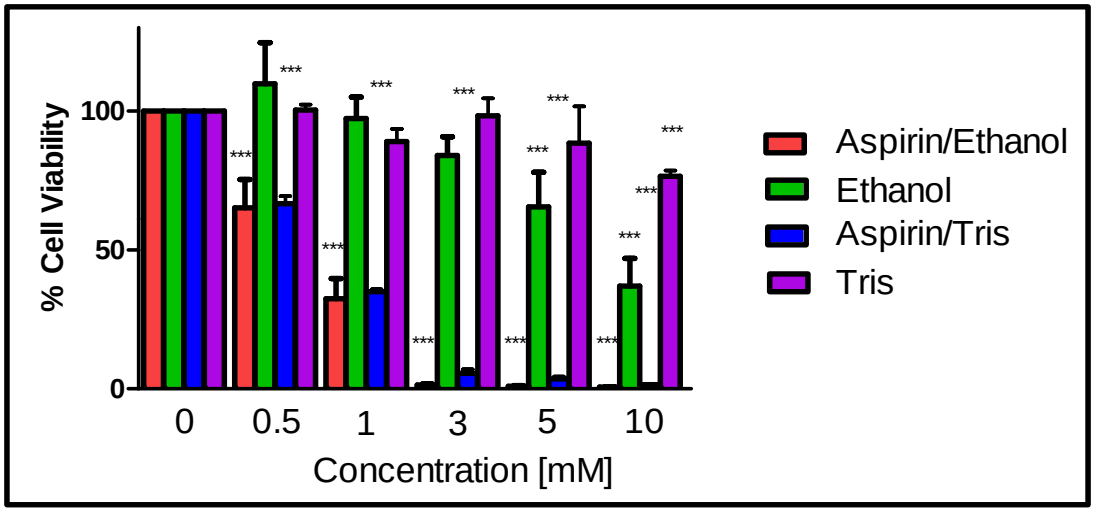
A 24 hour incubation with various concentrations of aspirin caused significant decreases in SW480 cell viability as assessed by MTT assay. Graphical representations of changes in cell viability were calculated using GraphPad Prism 5 software. All graphs are displayed as means ± S.E.M. from 3 experiments performed in quintuplicate. * denotes $P < 0.5$, ** denotes $P < 0.05$ and *** denotes $P < 0.005$ [One-Way ANOVA with Tukey's Multiple Comparison Test].

Figure 4.2. Cell viability of SW480 cells treated with aspirin for 48 hours.



A 48 hour incubation with various concentrations of aspirin caused significant decreases in SW480 cell viability as assessed by MTT assay. Graphical representations of changes in cell viability were calculated using GraphPad Prism 5 software. All graphs are displayed as means ± S.E.M. from 3 experiments performed in quintuplicate. ** denotes $P < 0.05$ and *** denotes $P < 0.005$ [One-Way ANOVA with Tukey's Multiple Comparison Test].

Figure 4.3. Cell viability of SW480 cells treated with aspirin for 72 hours.

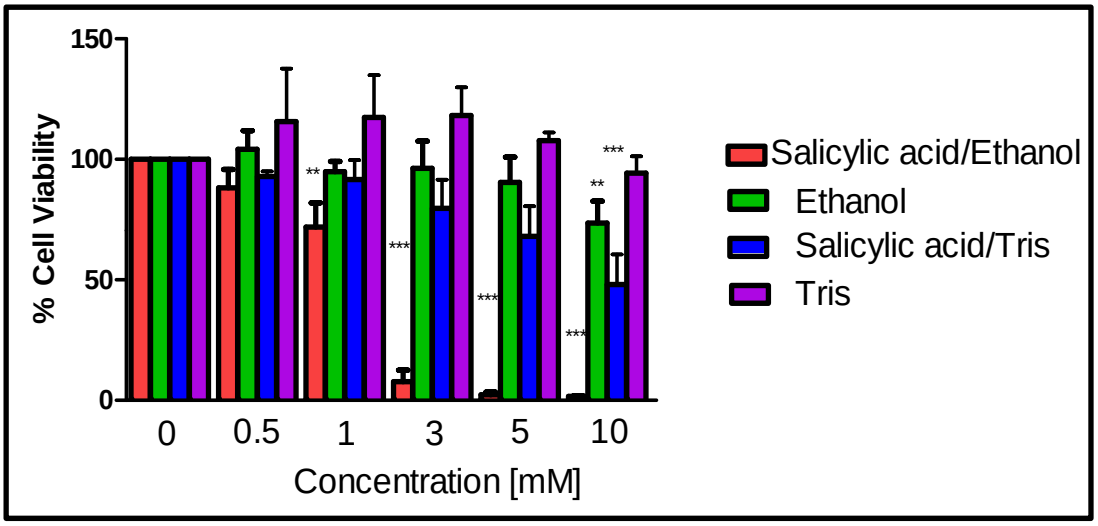


A 72 hour incubation with various concentrations of aspirin caused significant decreases in SW480 cell viability as assessed by MTT assay. Graphical representations of changes in cell viability were calculated using GraphPad Prism 5 software. All graphs are displayed as means ± S.E.M. from 3 experiments performed in quintuplicate. *** denotes $P < 0.005$ [One-Way ANOVA with Tukey's Multiple Comparison Test].

4.3.2 Salicylic acid affects SW480 cell viability

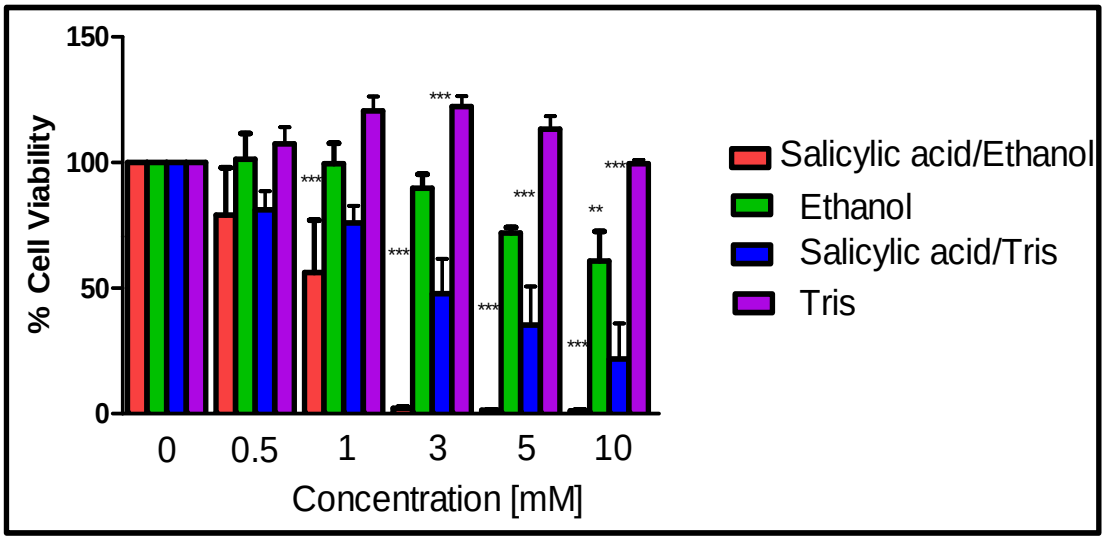
Cell viability assays were carried out on SW480 cells upon salicylic acid treatment in order to determine cytotoxicity. Cells were treated for 24 (Figure 4.4), 48 (Figure 4.5) and 72 hours (Figure 4.6) with a range of salicylic acid concentrations (0.5 – 10 mM). As with aspirin, salicylic acid also demonstrated a concentration-dependent decrease in SW480 cell viability and also a time-dependent decrease as indicated in Figures 4.4 – 4.6. Again, as with aspirin, the results show that the effects of salicylic acid on cell viability were dependent on the diluent used. After 24 hours, salicylic acid dissolved in ethanol caused almost total cell death at 24 hours at high concentrations i.e., 5 mM and 10 mM as shown in Figure 4.4 with total cell death observed at these concentrations and also at 3 mM at 48 hours onwards (Figures 4.5 and 4.6). However, salicylic acid dissolved in Tris.HCl buffer did not cause total cell death even at the high concentration utilised after 72 hours treatment (Figure 4.6).

Figure 4.4. Cell viability of SW480 cells treated with salicylic acid for 24 hours.



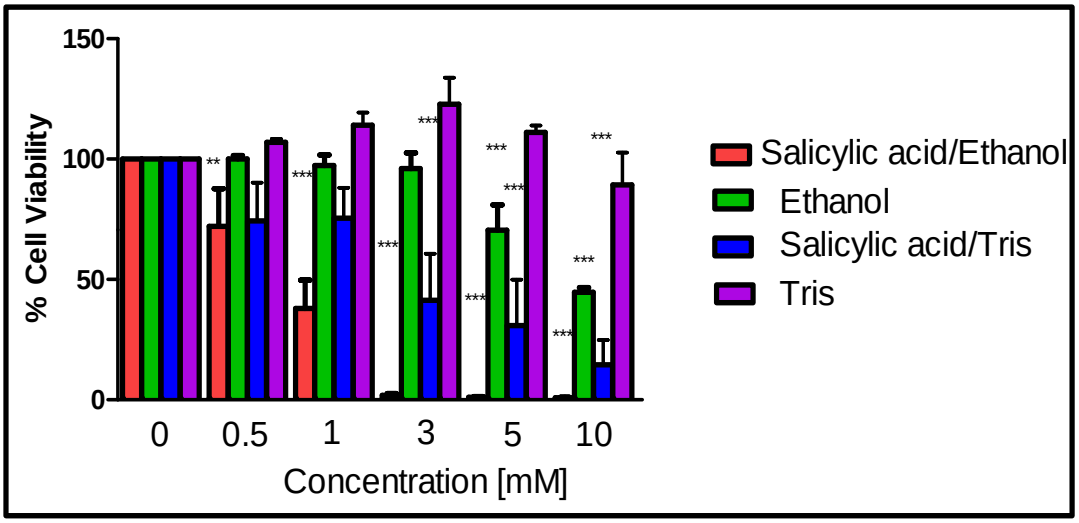
A 24 hour incubation with various concentrations of salicylic acid caused significant decreases in SW480 cell viability as assessed by MTT assay. Graphical representations of changes in cell viability were calculated using GraphPad Prism 5 software. All graphs are displayed as means ± S.E.M. from 3 experiments performed in quintuplicate. ** denotes $P < 0.05$ and *** denotes $P < 0.005$ [One-Way ANOVA with Tukey's Multiple Comparison Test].

Figure 4.5. Cell viability of SW480 cells treated with salicylic acid for 48 hours.



A 48 hour incubation with various concentrations of salicylic acid caused significant decreases in SW480 cell viability as assessed by MTT assay. Graphical representations of changes in cell viability were calculated using GraphPad Prism 5 software. All graphs are displayed as means ± S.E.M. from 3 experiments performed in quintuplicate. ** denotes $P < 0.05$ and *** denotes $P < 0.005$ [One-Way ANOVA with Tukey's Multiple Comparison Test].

Figure 4.6. Cell viability of SW480 cells treated with salicylic acid for 72 hours.



A 72 hour incubation with various concentrations of salicylic acid caused significant decreases in SW480 cell viability as assessed by MTT assay. Graphical representations of changes in cell viability were calculated using GraphPad Prism 5 software. All graphs are displayed as means ± S.E.M. from 3 experiments performed in quintuplicate. * denotes $P < 0.5$, ** denotes $P < 0.05$ and *** denotes $P < 0.005$ [One-Way ANOVA with Tukey's Multiple Comparison Test].

4.3.3 Aspirin affects cell viability of colorectal and non-colorectal cancer cell lines

Aspirin has been shown to be cytotoxic to colorectal cancer cells *in vitro* (Smith *et al.,* 2000; Stark *et al.,* 2001; Yin *et al.,* 2006; Luciani *et al.,* 2007). The effects of aspirin on cell viability were investigated for non-colorectal cancer cell lines including breast cancer cell lines MCF7 and MDA231-MB and glioma cell line U373MG and also another colorectal cancer cell line HCT116 for comparison and whether aspirin cytotoxicity was dependent on DNA MMR or p53 status (Table 4.1). Aspirin was diluted in Tris.HCl buffer (pH 7.2) as described in 'Materials and Methods' (Chapter 2, Section 2.1.6).

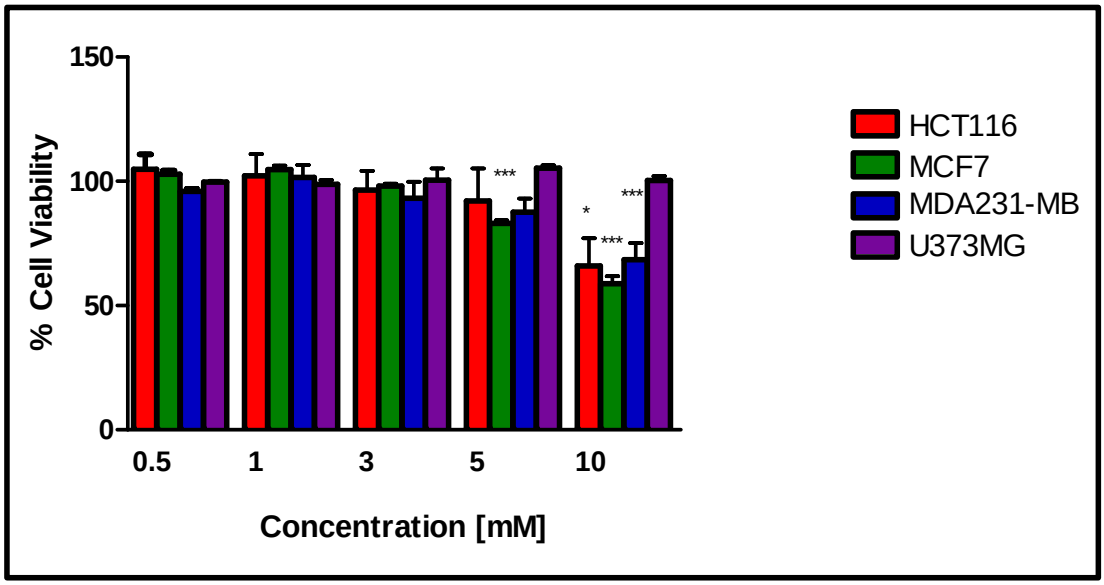
Table 4.1 DNA Mismatch repair status of cell lines used in cell viability assays

(Adapted from Fuxe *et al.,* 2000; Tentori *et al.,* 2002; Din *et al.,* 2004)

Cell Line	MMR Status	p53
SW480	Proficient	Mutant
HCT116	Deficient	Wild type
MCF7	Proficient	Wild type
MDA231-MB	Not known	Mutant
U373MG	Proficient	Mutant

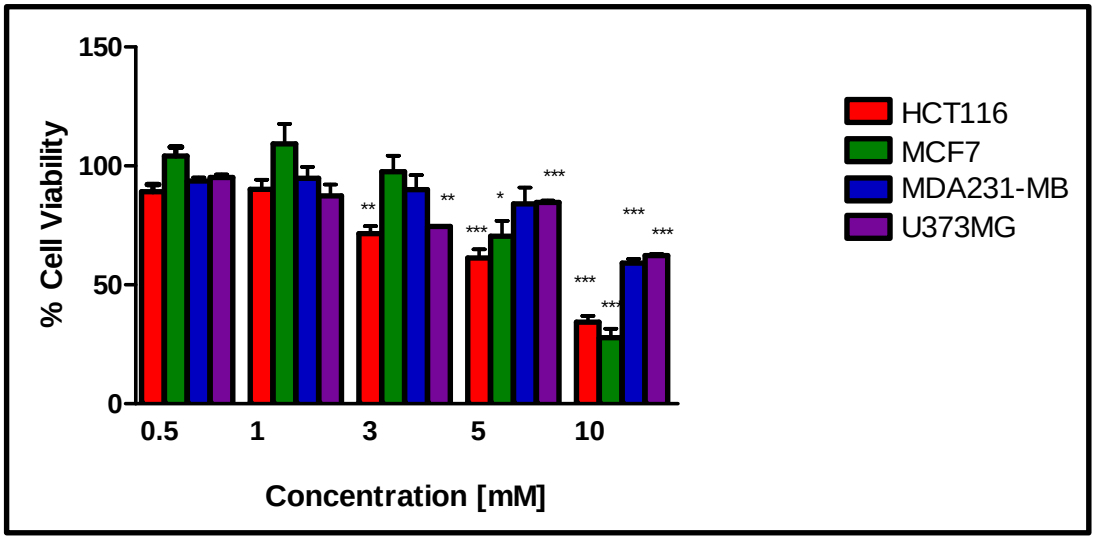
Interestingly, none of the cell lines in the panel except SW480 were affected at low concentrations, 0.5 and 1 mM aspirin even after 72 hour treatment (Figure 4.9). Also, HCT116 are less sensitive to aspirin treatment (Figure 4.7, Figure 4.8 and Figure 4.9) than SW480 cells (Figure 4.1, Figure 4.2 and Figure 4.3). SW480 sensitivity to aspirin compared to other colorectal cancer cell lines has previously been reported (Akashi *et al.,* 2000).

Figure 4.7. Cell viability of colorectal and non-colorectal cancer cell lines treated with aspirin for 24 hours.



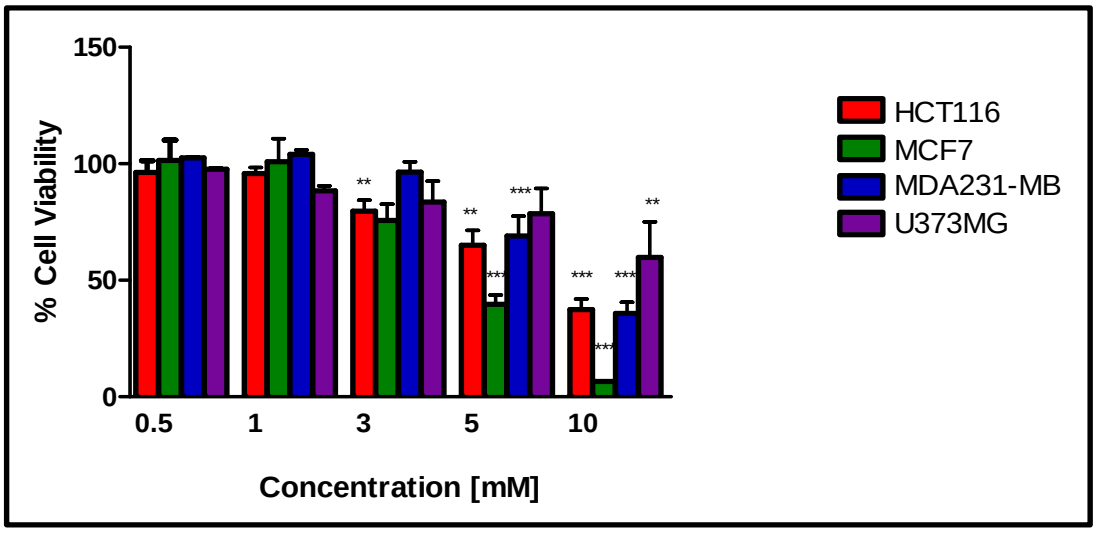
A 24 hour incubation with various concentrations of aspirin caused significant decreases in cancer cell viability as assessed by MTT assay. Graphical representations of changes in cell viability were calculated using GraphPad Prism 5 software. All graphs are displayed as means ± S.E.M. from 3 experiments performed in quintuplicate. * denotes $P < 0.5$, ** denotes $P < 0.05$ and *** denotes $P < 0.005$ [One-Way ANOVA with Tukey's Multiple Comparison Test].

Figure 4.8. Cell viability of colorectal and non-colorectal cancer cell lines treated with aspirin for 48 hours.



A 48 hour incubation with various concentrations of aspirin caused significant decreases in cancer cell viability as assessed by MTT assay. Graphical representations of changes in cell viability were calculated using GraphPad Prism 5 software. All graphs are displayed as means ± S.E.M. from 3 experiments performed in quintuplicate. * denotes $P < 0.5$, ** denotes $P < 0.05$ and *** denotes $P < 0.005$ [One-Way ANOVA with Tukey's Multiple Comparison Test].

Figure 4.9. Cell viability of colorectal and non-colorectal cancer cell lines treated with aspirin for 72 hours.



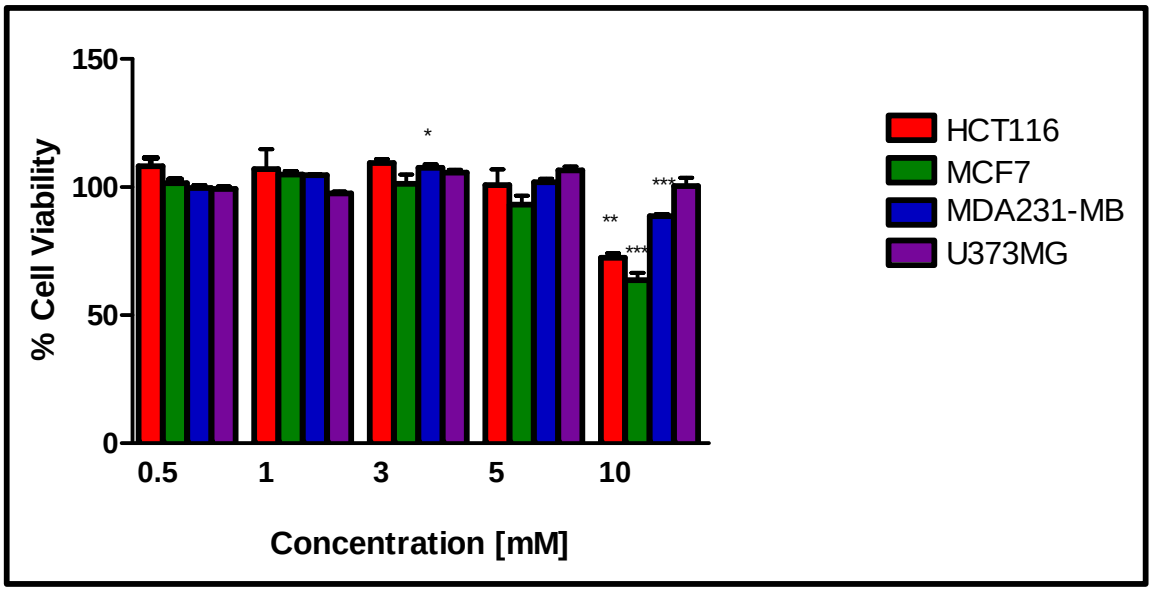
A 72 hour incubation with various concentrations of aspirin caused significant decreases in cancer cell viability as assessed by MTT assay. Graphical representations of changes in cell viability were calculated using GraphPad Prism 5 software. All graphs are displayed as means ± S.E.M. from 3 experiments performed in quintuplicate. * denotes $P < 0.5$, ** denotes $P < 0.05$ and *** denotes $P < 0.005$ [One-Way ANOVA with Tukey's Multiple Comparison Test].

4.3.4 Salicylic acid affects cell viability of colorectal and non-colorectal cancer cell lines

In addition to analysing aspirin cytotoxicity to non-colorectal cancer cell lines, it was decided to analyse salicylic acid cytotoxicity to these cell lines. Salicylic acid was diluted in Tris.HCl buffer (pH 7.2) as described in 'Materials and Methods (Chapter 2, Section 2.1.6). The results show (Figures 4.10 – 4.12) that like the effects of salicylic acid and also aspirin on cell viability of SW480 cells, salicylic acid affected cell proliferation particularly at the highest concentrations (10 mM) of all cell lines.

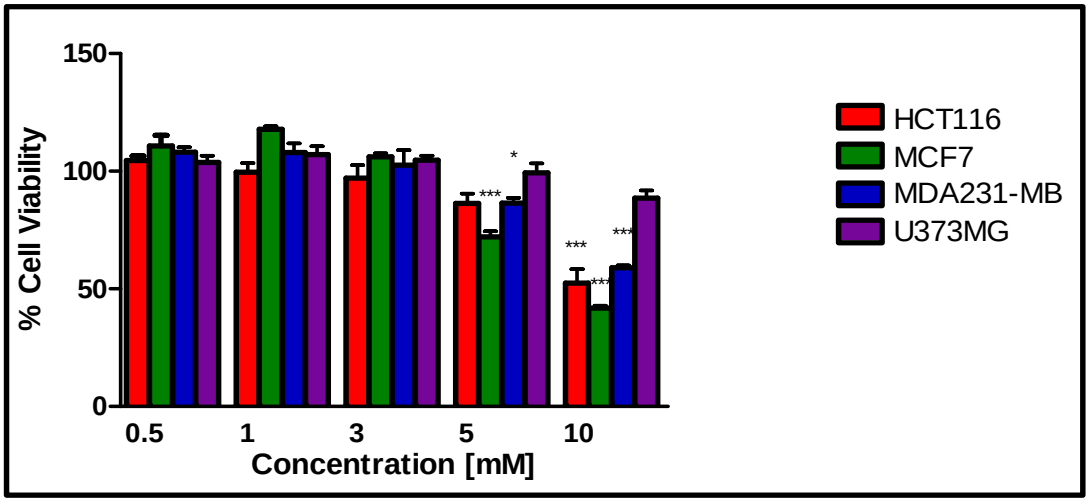
MCF7 cell viability was most significantly altered by incubation with 10 mM salicylic acid treatment for 72 hours (Figure 4.12) than any other cell line including HCT116. Like with aspirin, salicylic acid did not have any significant effect on cell viability at low concentrations (1 – 3 mM) even after 72 hour treatment (Figures 4.10 – 4.12).

Figure 4.10. Cell viability of colorectal and non-colorectal cancer cell lines treated with salicylic acid for 24 hours.



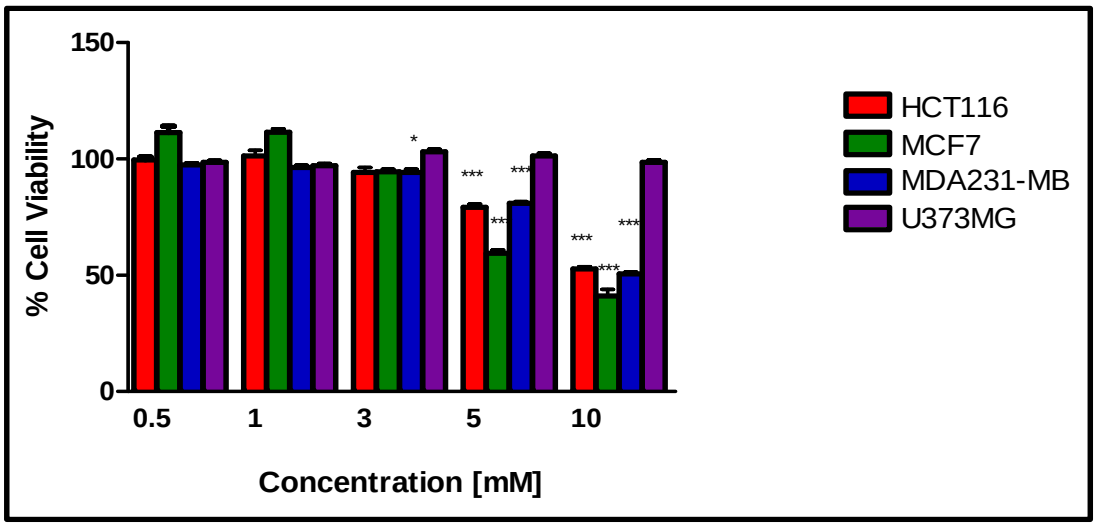
A 24 hour incubation with various concentrations of salicylic acid caused significant decreases in cancer cell viability as assessed by MTT assay. Graphical representations of changes in cell viability were calculated using GraphPad Prism 5 software. All graphs are displayed as means ± S.E.M. from 3 experiments performed in quintuplicate. * denotes $P < 0.5$, ** denotes $P < 0.05$ and *** denotes $P < 0.005$ [One-Way ANOVA with Tukey's Multiple Comparison Test].

Figure 4.11. Cell viability of colorectal and non-colorectal cancer cell lines treated with salicylic acid for 48 hours.



A 48 hour incubation with various concentrations of salicylic acid caused significant decreases in cancer cell viability as assessed by MTT assay. Graphical representations of changes in cell viability were calculated using GraphPad Prism 5 software. All graphs are displayed as means ± S.E.M. from 3 experiments performed in quintuplicate. * denotes $P < 0.5$, ** denotes $P < 0.05$ and *** denotes $P < 0.005$ [One-Way ANOVA with Tukey's Multiple Comparison Test].

Figure 4.12. Cell viability of colorectal and non-colorectal cancer cell lines treated with salicylic acid for 72 hours.



A 72 hour incubation with various concentrations of salicylic acid caused significant decreases in cancer cell viability as assessed by MTT assay. Graphical representations of changes in cell viability were calculated using GraphPad Prism 5 software. All graphs are displayed as means ± S.E.M. from 3 experiments performed in quintuplicate. * denotes $P < 0.5$, ** denotes $P < 0.05$ and *** denotes $P < 0.005$ [One-Way ANOVA with Tukey's Multiple Comparison Test].

These cell viability experiments were carried out to determine appropriate aspirin concentration, appropriate aspirin diluents and also an appropriate treatment time frame for subsequent investigations in this chapter.

Previous research has suggested that approximately 1 mM aspirin is physiologically relevant (Stark *et al*., 2001). Also Goel *et al*., (2003) have shown that 1 mM aspirin treatment causes a significant increase in MLH1 protein expression. Figure 4.2 has shown that 1 mM aspirin does have a significant effect on SW480 cell viability from 48 hours onwards.

A 48 hour incubation period with aspirin was selected for further investigation for the following reasons: cell viability results indicated that 48 hour treatments with 1 mM aspirin caused a significant decrease in cell viability of SW480 cells (Figure 4.2) whereas 24 hour treatment did not cause a significant decrease in cell viability (Figure 4.1) and also Stark *et al.,* (2001) reported that significant nuclear translocation of NFκB was possible in SW480 cells at 48 hr incubation at a concentration of 1 mM. It was also shown by Goel *et al.,* (2003) that apoptosis appeared to peak in SW480 cells after 48 hour treatment with aspirin. Figure 4.2 also shows that the diluent (Tris.HCl buffer) had no significant effect on cell viability at 1 mM concentration.

Based on the preliminary optimisation carried out and as a consequence of previously published research it was decided that for subsequent analysis aspirin would be dissolved in Tris.HCl buffer and that SW480 cells would be treated with 1 mM aspirin for 48 hours.

4.4 Analysis of MBD4 expression in colorectal cancer cell line SW480 following aspirin and salicylic acid treatment

MLH1 and MBD4 are reported to be transcriptionally coupled and physically interact to form a complex which can be immunoprecipitated (Bellacosa *et al.,* 1999) and as *MLH1* expression increases with aspirin treatment (Goel *et al.,* 2003) it would be reasonable to expect that *MBD4* expression may also increase upon aspirin treatment. To test this hypothesis, semi-quantitative PCR and quantitative real-time PCR analysis were carried out to determine any changes to *MBD4* mRNA expression upon aspirin treatment.

The results presented demonstrate that although no significant change in *MBD4* gene expression upon 48 hour treatment with 1 mM aspirin or 1 mM salicylic acid compared to control was observed with semi-quantitative PCR analysis, a significant increase in *MBD4* gene expression upon salicylic acid was observed with quantitative real-time PCR analysis.

4.4.1 Semi-Quantitative PCR analysis

Semi-quantitative PCR was carried out as described in 'Materials and Methods' (Chapter 2, Section 2.2.3). Briefly, semi-quantitative PCR products were electrophoresed on a 3% agarose gel with ethidium bromide and visualised using UV light.

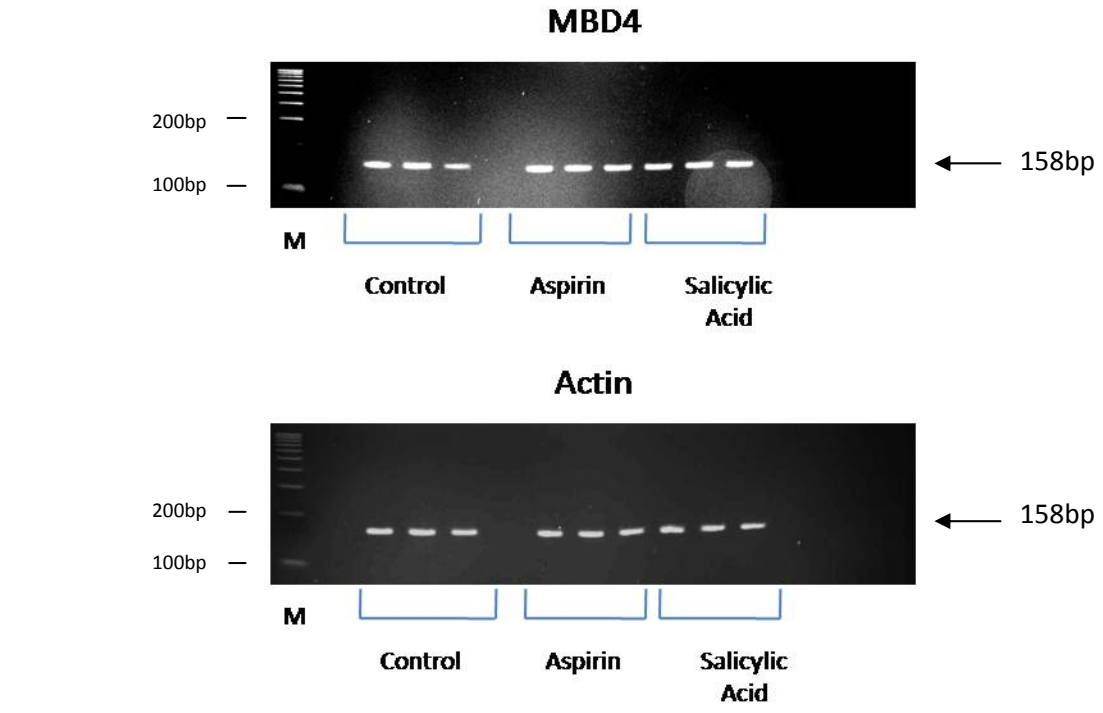
Image data was analysed using Scion Image Software (http://www.scioncorp.com). Signal intensities were quantified by measuring the mean pixel intensity of bands of interest and values were expressed as gene of interest over reference gene.

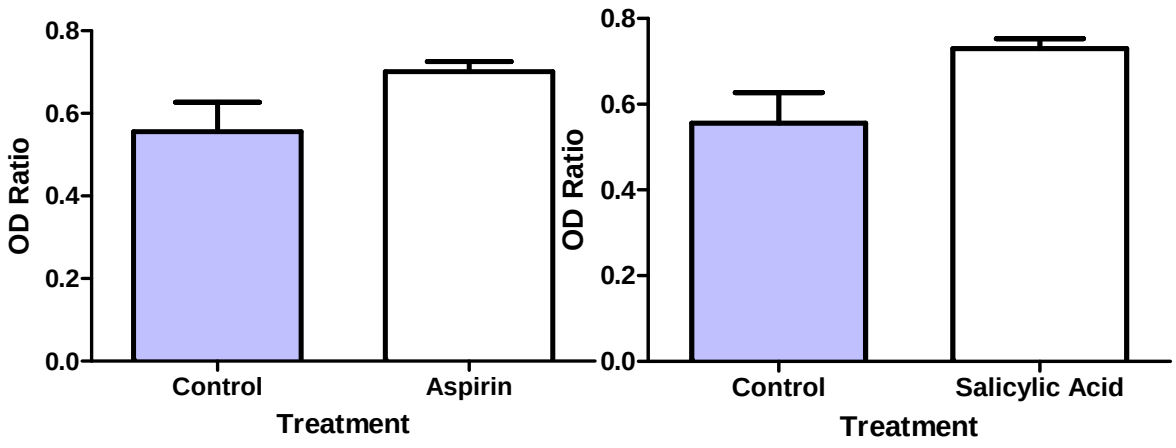
Semi-quantitative PCR evaluation of *MBD4* mRNA expression showed that when SW480 cells were incubated with 1mM aspirin for 48 hours, no significant effect (Figure 4.13) compared to untreated samples was seen ($P > 0.5$ for both aspirin and salicylic acid).

Figure 4.13. Semi-quantitative PCR analysis of *MBD4* gene expression upon treatment with 1 mM aspirin and 1 mM salicylic acid for 48 hours.

Incubation of SW480 cells for 48 hours with 1 mM aspirin and 1 mM salicylic acid had a small though non significant effect on *MBD4* mRNA expression compared to control (treated with equivalent amount of Tris.HCl buffer) samples.

The optical density of the bands visualised by gel electrophoresis was measured by Scion Image analysis. Optical density values of the bands obtained for *MBD4* were normalised to *actin*. The graphs display the optical density ratio of *MBD4/actin*. Significant difference in optical density was measured using an unpaired t-test. (P = 0.1262 and P = 0.0812 for aspirin and salicylic acid respectively). n = 3 and a representative gel image is shown in the figure. M = 100bp maker





4.4.2 Quantitative Real-Time PCR analysis

For a more sensitive analysis of gene expression, quantitative real-time PCR evaluation of *MBD4* mRNA expression was carried out.

For quantitative real-time PCR, SYBR Green chemistry was utilised. SYBR Green is an intercalating dye, much like ethidium bromide, which binds to double stranded DNA and once bound emits fluorescence. As a consequence this fluorescence can be used to monitor amount of amplification product per PCR cycle, because as amplification product theoretically doubles with every cycle, a concomitant increase in SYBR Green fluorescence should also be seen. Measured fluorescence can be plotted against PCR cycle giving an amplification graph allowing for a 'real time' analysis of mRNA expression per cycle. PCR amplification is exponential and hence quantitative real-time PCR allows for relative amounts of amplification product to be determined.

The cycle threshold or Ct value is the PCR cycle number at which the fluorescence signal is greater than the minimal detection level and as the Ct value is directly proportional to the amount of starting template it is used to calculate mRNA expression levels. To calculate the cycle difference or ΔCt between two samples, the Ct value of one sample (e.g., treated sample) is subtracted from the Ct value of another sample (e.g., control sample). As PCR is exponential, this ΔCt value can be converted into a linear form by 2^{-}(ΔCt) and this is used for quantitative relative expression of mRNA (Ginzinger., 2002).

However, as SYBR Green is a non-specific intercalating dye and binds to any double stranded DNA, it can also bind to primer dimers and any contaminating genomic DNA which results in a false increase in fluorescence. For this reason melt curve analysis is carried out to determine specificity. After amplification the temperature is raised in 0.5 ^{o}C increments, the fluorescence is detected and a melt curve is generated.

An initial study was carried out to determine which reference genes would be appropriate to use in this experimental model. For accurate normalisation of data it is essential that reference genes are chosen which are stably expressed in the experimental system, i.e., the genes used to normalise the results are not themselves affected by the experimental conditions. For this reason, geNorm analysis was carried out as described in 'Materials and Methods' (Chapter 2,

Section 2.2.5) and showed that out of a panel of 12 reference genes, the two most stably expressed genes were *GAPDH* and *CYC1* and pairwise variation determined that the addition of the third most stably expressed gene *UBC* would be optimal for normalisation (Figures 4.14 and 4.15). These three genes were then used in subsequent Quantitative real-time PCR analysis.

As shown in Figure 4.16 no significant difference in *MBD4* gene expression was found upon aspirin treatment for 48 hours (P = 0.4829). However, interestingly *MBD4* expression was significantly increased when treated with 1 mM salicylic acid for 48 hours (P = 0.0260) as shown in Figure 4.17.

It is interesting to note that the significance of the data for salicylic acid was dependent upon the house keeping genes used highlighting the importance of appropriate selection of these genes. When *MBD4* expression was analysed using only *actin* as a reference gene the statistical difference in gene expression was not of significance (data not shown), however, when the data was analysed against the reference genes determined from the geNorm analysis then the data became statistically significant.

4.4.2.1 geNorm analysis to determine appropriate reference genes

In order to determine the most appropriate reference genes for the experimental model the stability of expression of a panel of reference genes was evaluated using geNorm analysis (Vandesompele *et al.,* 2002) as described in 'Materials and Methods' (Chapter 2, Section 2.2.5). Data was analysed using geNorm software provided with the kit (PrimerDesign, Southampton, UK).

Two parameters are defined to quantify the reference gene stability: *M* (average expression stability) and *V* (pairwise variation) (Vandesompele *et al.,* 2002). The more stably expressed the gene is, the lower the *M* value, and therefore the more suitable that particular gene is for use as a control. For pairwise variation, Vandesompele *et al.* (2002) proposed 0.15 as a cut-off value below which the inclusion of an additional control gene is not required.

The results rank the panel of genes analysed according to *M* value and indicated that in this experimental model the two reference genes suitable were *GAPDH* and *CYC1* as shown in Figure 4.14. In addition pairwise variation indicated that the inclusion of the third most stably expressed

reference gene *UBC* could also be used as an additional control gene for optimal normalisation as shown in Figure 4.15.

Figure 4.14. Average expression stability values of reference genes. The panel of 12 genes included in the geNorm kit (PrimerDesign Ltd, Southampton, UK) have been ranked according to expression stability with the least stable at the left and the most stable at the right. The least stably expressed gene was *ATP5B* and the two most stable genes are *GAPDH* and *CYC1*.

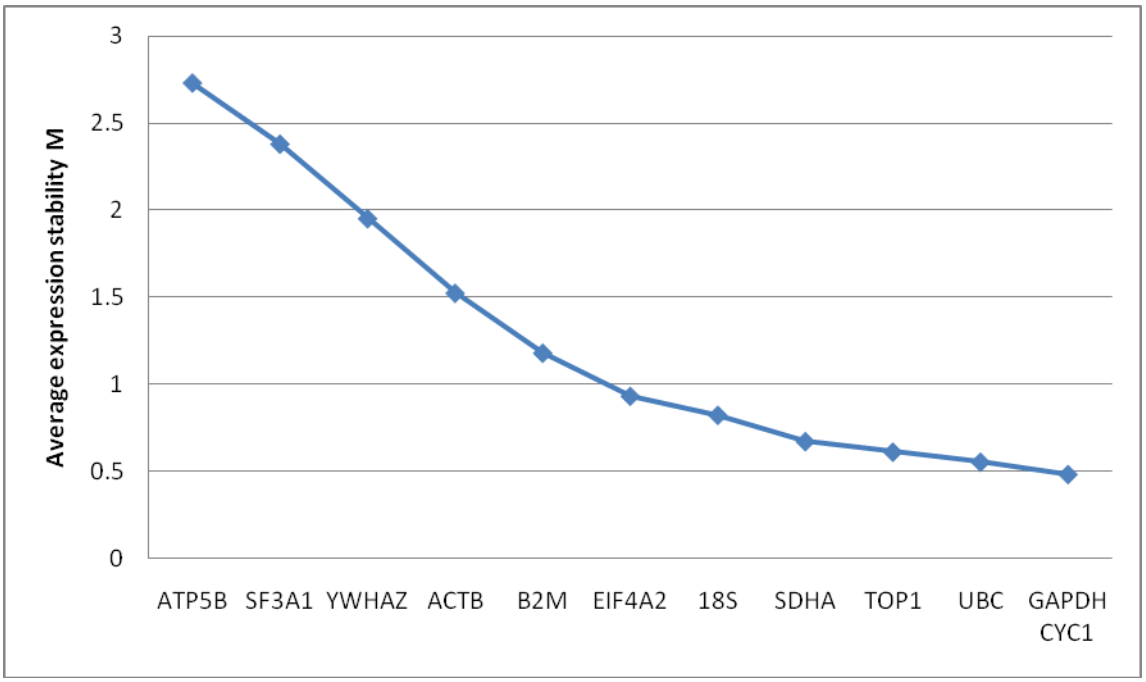


Figure 4.15. Determination of the optimal number of control genes for normalisation. Pairwise variation (*V*) was determined for the panel of 12 genes included in the geNorm kit (PrimerDesign Ltd, Southampton, UK). A *V* score of below 0.5 is recommended and the results show that a *V* score of 0.147 is achieved with three reference genes, so the average top three stably expressed genes would be the optimal normalisation factor for this experimental model.

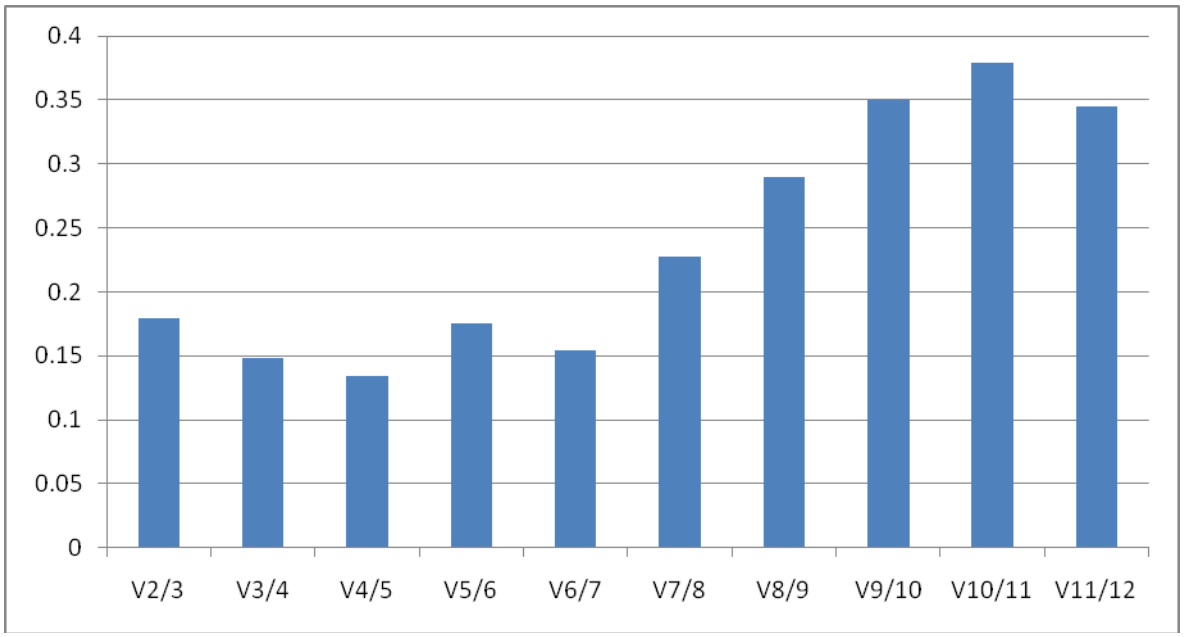


Figure 4.16. Quantitative real-time PCR analysis of *MBD4* gene expression upon treatment with 1mM aspirin and 1 mM salicylic acid for 48 hours. The graphs display the relative expression of *MBD4* to reference genes as calculated by comparison of Ct values (Δ – Δ Ct). Significant difference in relative expression was measured using an unpaired t-test. Results are displayed as means ± S.E.M. from 3 experiments performed in triplicate.

(A) Incubation of SW480 cells for 48 hours with 1 mM aspirin does not cause a significant increase of *MBD4* mRNA expression compared to control (treated with equivalent amount of Tris-HCl buffer) samples. n = 3 with each experiment run in triplicate. $P > 0.05$.

(B) Incubation of SW480 cells for 48 hours with 1 mM salicylic acid caused a significant increase on *MBD4* mRNA expression compared to control (treated with equivalent amount of Tris-HCl buffer) samples. n = 3 with each experiment run in triplicate. $P < 0.05$ as indicated by *

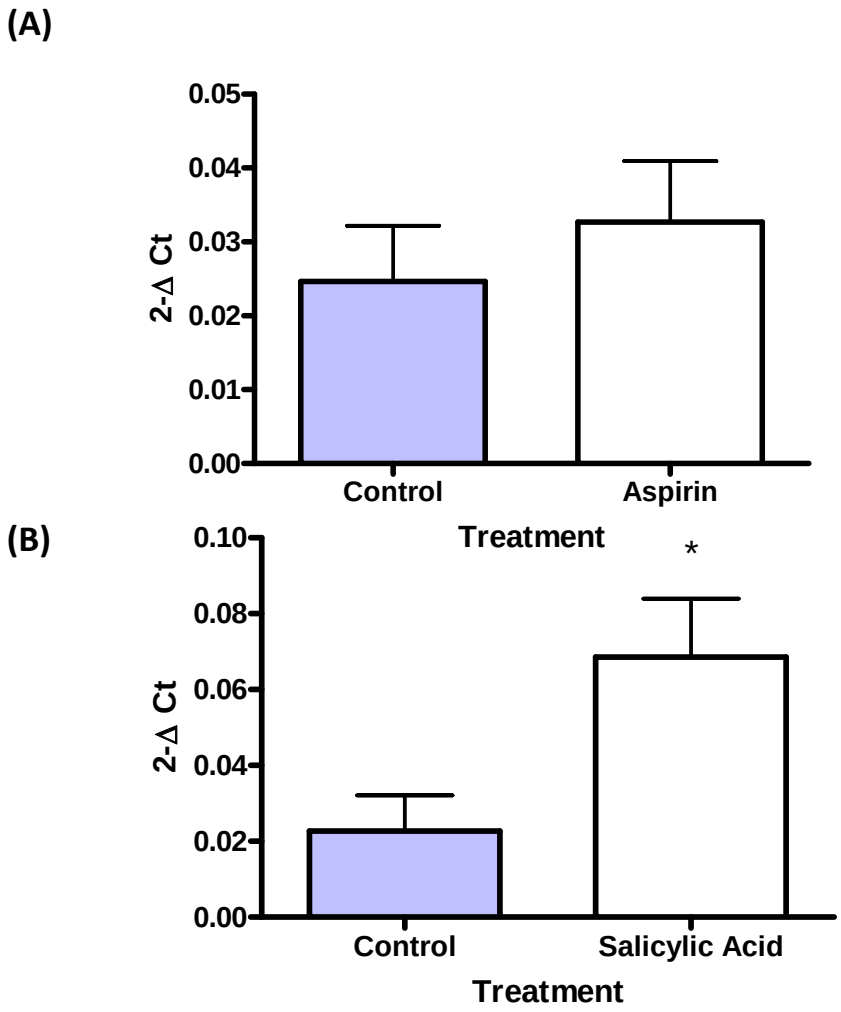
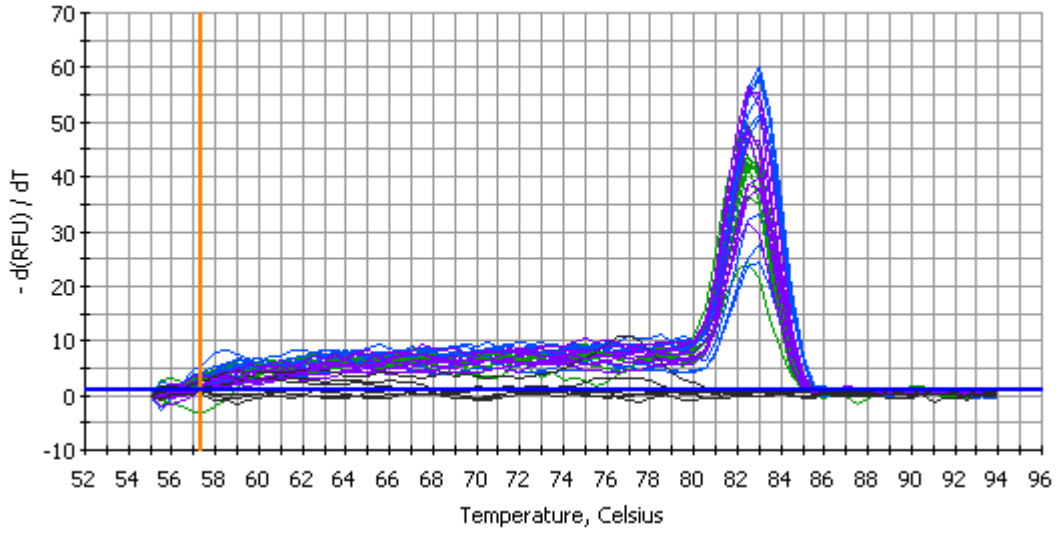
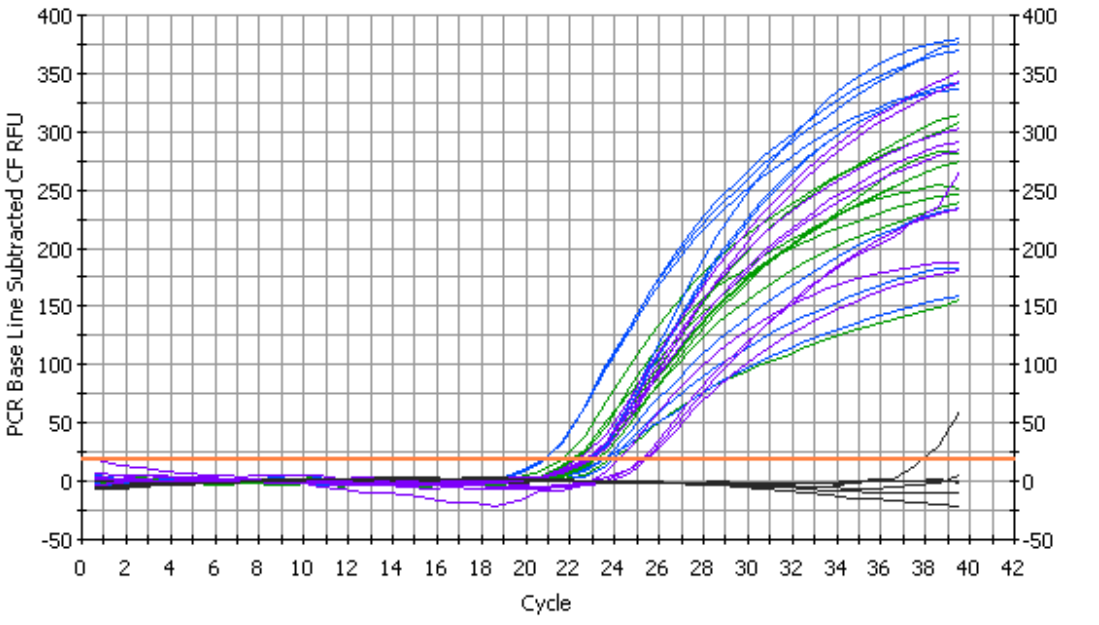


Figure 4.17. Amplification plot and melt curve analysis of the *MBD4* primer set. (A) The melt curve shows a single peak indicating primer specificity. **(B)** The different traces indicate the different reactions carried out in triplicate in individual wells. Each experiment was carried out 3 times and a representative image is shown. The black traces represent the negative controls.

(A)



(B)



4.5 Analysis of DNA repair genes expression in colorectal cancer cell line SW480 following aspirin treatment utilising RT^2 Profiler^{TM} PCR Array.

Although semi-quantitative and quantitative real-time PCR showed that aspirin appeared to have no significant affect on *MBD4* gene expression (as shown in Figures 4.13 and 4.16), it was decided to test the effects of aspirin on other DNA repair genes. DNA mismatch repair proteins have been shown to be affected by aspirin treatment (Goel *et al.,* 2003) and may play a part in the mechanism of action of aspirin cytotoxicty. Hardwick *et al*., (2004) also showed that aspirin induces genes involved with DNA damage signalling as well as nucleotide metabolism and the stress response. Due to such alterations in the expression of DNA repair genes and in light of the likely long term nature of aspirin treatment as a chemoprotective agent, it was reasoned that it would be informative with respect to understanding the effect of the use of aspirin on colorectal cell genome stability to re-examine DNA repair gene expression as a consequence of the availability of a PCR array dedicated for DNA repair gene expression, and exposing cells to physiologically achievable levels (1 mM) of aspirin.

To investigate whether aspirin affected expression of genes involved in DNA damage signalling a commercially available RT^2 Profiler^{TM} PCR Array (SuperArray Biosciences Corporation, Frederick, MD) was utilised to assess the expression of 84 different genes involved in DNA damage signalling pathways upon aspirin treatment. The benefit of this method is that a multiple number of different genes (in this case 84) can be analysed at the same time with the same sample.

The results presented here (e.g., Figure 4.18, Table 4.1) show that PCR array analysis demonstrated that a 48-hour incubation of SW480 cells with 1 mM aspirin caused marked decreases in mRNA expression of several key genes involved in DNA damage signalling pathways including significant decreases in *BRCA1* (fold change = 0.15; *P* value = 0.04), *ATR* (fold change = 0.24; *P* value = 0.001) and *MAPK12* (fold change = 0.41; *P* value = 0.05) and a significant increase in *XRCC3* (fold change = 7.37; *P* value = 0.05).

Table 4.2. Comparison of the transcriptional level of 84 genes important for DNA damage signalling pathways analysed by RT^2 Profiler^{TM} PCR Array.

Data was analyzed with the Excel-based PCR Array Data Analysis Template downloaded from the SuperArray website (www.sabiosciences.com) and normalised to the expression level of reference control genes provided on the arrays.

Genes highlighted are those which showed a significant fold difference in gene expression ($P < 0.05$). *GADD45α* is also highlighted as although not of statistical significance the large fold increase in gene transcription (x 14.78) is of interest.

Accession Number	*Gene Name*	*T-Test P-Value*	*Fold Difference (Treated/Untreated)*
Hs. 431048	*ABL1*	0.14	3.03
Hs.601206	*ANKRD17*	0.16	0.30
Hs.73722	*APEX1*	0.42	0.73
Hs.367437	*ATM*	0.46	0.59
Hs.271791	*ATR*	0.0014	0.24
Hs.533526	*ATRX*	0.83	1.29
Hs.194143	*BRCA1*	0.04	0.15
Hs.519162	*BTG2*	0.46	1.84
Hs.292524	*CCNH*	0.08	0.09
Hs.184298	*CDK7*	0.34	0.63
Hs.24529	*CHEK1*	0.19	0.52
Hs.291363	*CHEK2*	0.40	0.57

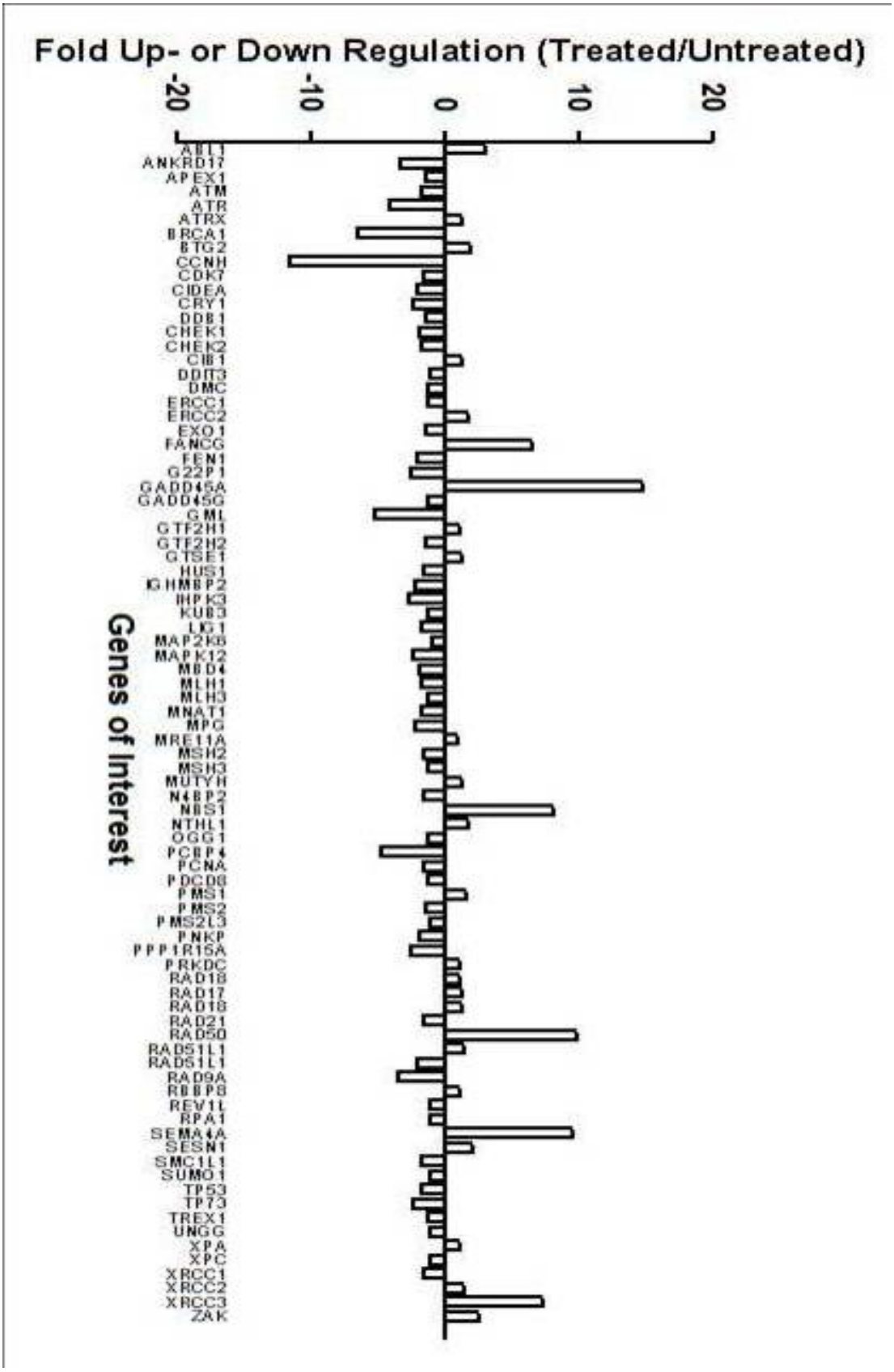
Hs.135471	*CIB1*	0.54	1.28
Hs.249149	*CIDEA*	0.48	0.48
Hs.151573	*CRY1*	0.10	0.42
Hs.290758	*DDB1*	0.37	0.72
Hs.505777	*DDIT3*	0.71	0.83
Hs.339396	*DMC1*	0.74	0.81
Hs.435981	*ERCC1*	0.74	0.82
Hs.487294	*ERCC2*	0.21	1.79
Hs.498248	*EXO1*	0.34	0.70
Hs.591084	*FANCG*	0.26	6.45
Hs.409065	*FEN1*	0.08	0.48
Hs.292493	*G22P1*	0.41	0.40
Hs.80409	*GADD45α*	0.16	14.78
Hs.9701	*GADD45λ*	0.77	0.75
Hs.545196	*GML*	0.17	0.19
Hs.577202	*GTF2H1*	0.84	1.09
Hs.191356	*GTF2H2*	0.62	0.67
Hs.386189	*GTSE1*	0.83	1.22
Hs.152983	*HUS1*	0.63	0.64
Hs.503048	*IGHMBP2*	0.23	0.45
Hs.17253	*IHPK3*	0.62	0.38
Hs.61188	*KUB3*	0.60	0.79

Hs.1770	*LIG1*	0.17	0.55
Hs.463978	*MAP2K6*	0.98	0.99
Hs.432642	*MAPK12*	0.05	0.41
Hs.35947	*MBD4*	0.18	0.52
Hs.195364	*MLH1*	0.56	0.59
Hs.436650	*MLH3*	0.53	0.81
Hs.509523	*MNAT1*	0.61	0.58
Hs.459596	*MPG*	0.47	0.46
Hs.192649	*MRE11A*	0.97	1.02
Hs.597656	*MSH2*	0.23	0.65
Hs.280987	*MSH3*	0.71	0.77
Hs.271353	*MUTYH*	0.84	1.22
Hs.396494	*N4BP2*	0.71	0.63
Hs.492208	*NBS1*	0.22	8.14
Hs.66196	*NTHL1*	0.39	1.77
Hs.380271	*OGG1*	0.63	0.79
Hs.20930	*PCBP4*	0.11	0.21
Hs.147433	*PCNA*	0.28	0.64
Hs.424932	*PDCD8*	0.71	0.80
Hs.111749	*PMS1*	0.49	1.58
Hs.632637	*PMS2*	0.11	0.66
Hs.225784	*PMS2L3*	0.69	0.86

Hs.78016	*PNKP*	0.60	1.97
Hs.631593	*PPP1R15A*	0.33	0.40
Hs.491682	*PRKDC*	0.86	1.16
Hs.531879	*RAD1*	0.86	1.10
Hs.16184	*RAD17*	0.65	1.30
Hs.375684	*RAD18*	0.87	1.21
Hs.81848	*RAD21*	0.27	0.65
Hs.128904	*RAD50*	0.16	9.79
Hs.631709	*RAD51*	0.83	1.42
Hs.172587	*RAD51L1*	0.07	0.47
Hs.240457	*RAD9A*	0.11	0.28
Hs.546282	*RBBP8*	0.94	1.03
Hs.443077	*REV1L*	0.84	0.84
Hs.461925	*RPA1*	0.89	0.95
Hs.408846	*SEMA4A*	0.18	9.55
Hs.591336	*SESN1*	0.46	2.00
Hs.211602	*SMC1L1*	0.22	0.55
Hs.81424	*SUMO1*	0.91	0.92
Hs.408312	*TP53*	0.17	0.57
Hs.192132	*TP73*	0.65	0.43
Hs.344812	*TREX1*	0.82	0.74
Hs.191334	*UNG*	0.63	0.87
Hs.591907	*XPA*	0.85	1.09

Hs.475538	*XPC*	0.86	0.94
Hs.98493	*XRCC1*	0.30	0.61
Hs.647093	*XRCC2*	0.78	1.42
Hs.592325	*XRCC3*	0.05	7.37
Hs.444451	*ZAK*	0.32	2.46

Figure 4.18. Graphical representation of Table 4.2. Graph showing fold up or down regulation of gene transcription after treatment with 1 mM aspirin for 48 hours compared to untreated samples as analysed by RT^2 Profiler^{TM} PCR Array.



4.5.1 Validation of RT^2 Profiler^{TM} PCR Array data

In order to validate the results from the PCR array, independent quantitative real-time PCR analysis was carried out on the genes of interest, *ATR*, *BRCA1*, *XRCC3* and *GADD45α* as described in 'Materials and Methods'. The reference genes selected by geNorm analysis (*GAPDH*, *CYC1* and *UBC*) were utilised. *MAPK12* was not chosen as a gene to validate as although the *P* value was 0.05, it showed a less than 4-fold difference in expression.

ATR gene expression was shown to be decreased upon 1 mM aspirin treatment for 48 hours compared to control by RT^2 Profiler^{TM} PCR Array, however quantitative real-time PCR validation showed no significant difference in gene expression (Figure 4.19). *BRCA1* gene expression was shown to be decreased upon 1 mM aspirin treatment for 48 hours compared to control by RT^2 Profiler^{TM} PCR Array, however quantitative real-time PCR validation showed no significant difference in gene expression (Figure 4.21). *XRCC3* gene expression was found to be significantly increased upon 1 mM aspirin treatment for 48 hours compared to control by RT^2 Profiler^{TM} PCR Array and this result was validated by quantitative real-time PCR (Figure 4.23). RT^2 Profiler^{TM} PCR Array analysis showed a large fold increase in *GADD45α* gene expression upon 1 mM aspirin treatment for 48 hours compared to control, however this increase was found to be non-significant. In contrast quantitative real-time PCR analysis showed this increase in gene expression to be statistically significant (Figure 4.25).

Figure 4.19. 48 hour incubation with 1 mM aspirin caused no significant difference in *ATR* mRNA expression compared to control

The graph displays the relative expression of *ATR* to reference genes as calculated by comparison of Ct values (Δ – Δ Ct). Significant difference in relative expression was measured using an unpaired t-test. Results are displayed as means ± S.E.M. from 3 experiments performed in triplicate. (*P* = 0.8907)

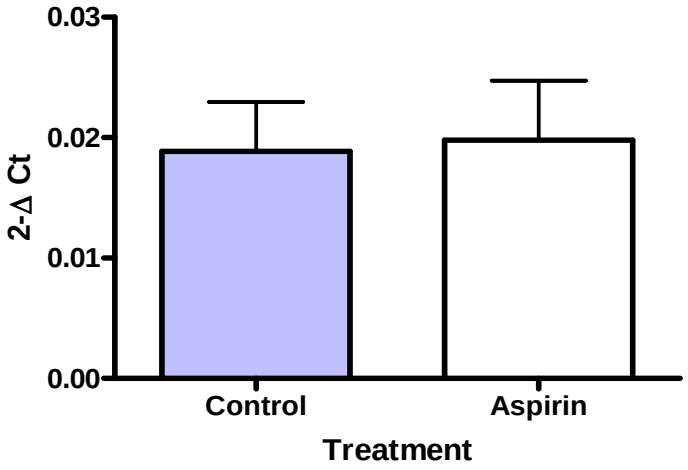
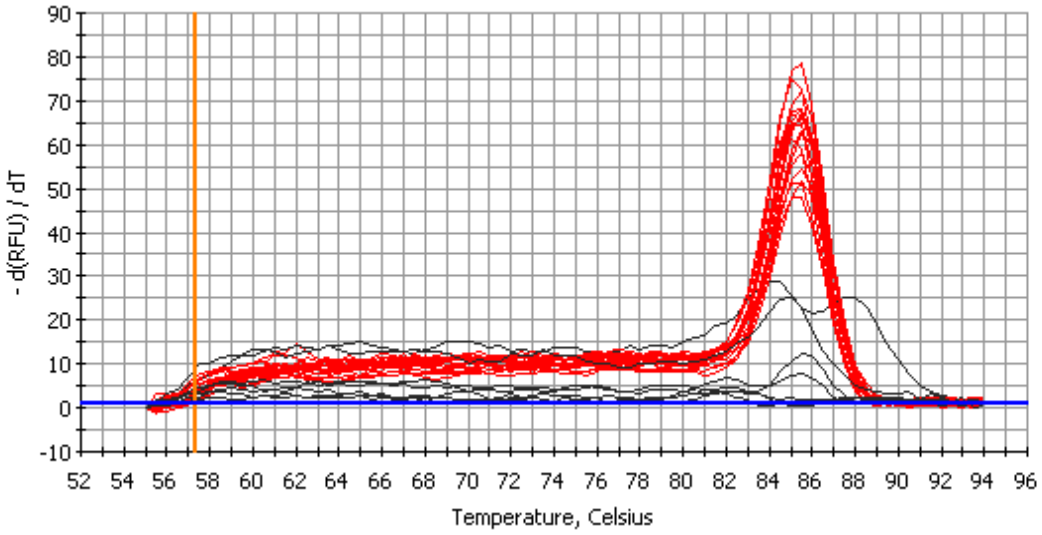


Figure 4.20 Amplification plot and melt curve analysis of *ATR* primer. (A) The melt curve shows a single peak indicating primer specificity. **(B)** Untreated and aspirin treated samples were run in triplicate in individual wells as indicated by the red traces and the black traces indicate negative control reactions. Each experiment was carried out 3 times and a representative image is shown.

(A)



(B)

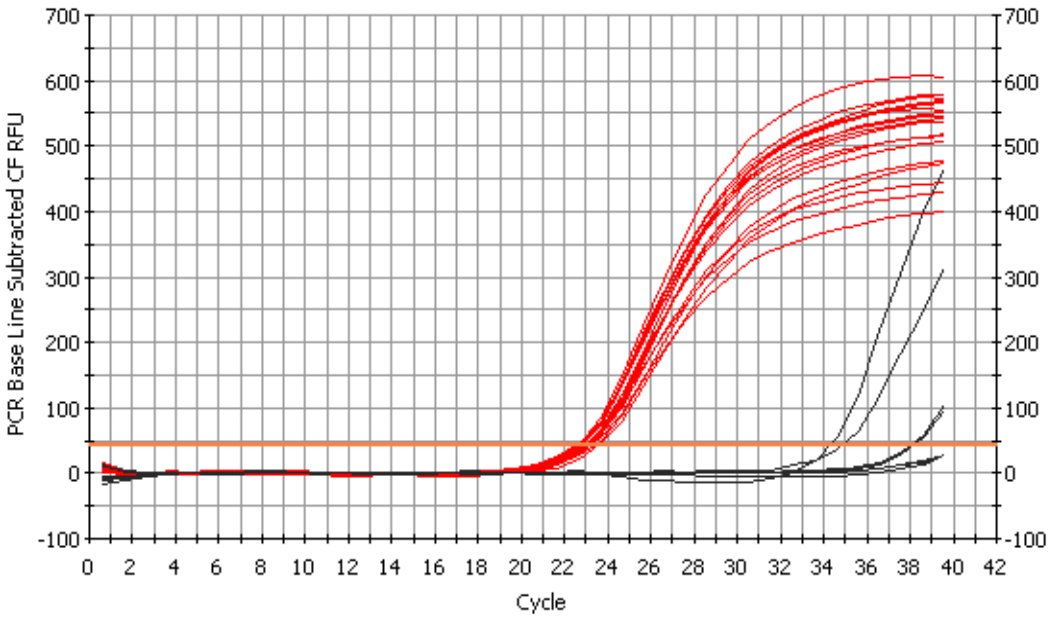


Figure 4.21. 48 hour incubation with 1 mM aspirin caused no significant difference in *BRCA1* mRNA expression compared to control.

The graph displays the relative expression of *BRCA1* to reference genes as calculated by comparison of Ct values (Δ – Δ Ct). Significant difference in relative expression was measured using an unpaired t-test. Results are displayed as means ± S.E.M. from 3 experiments performed in triplicate. (*P* = 0.5702)

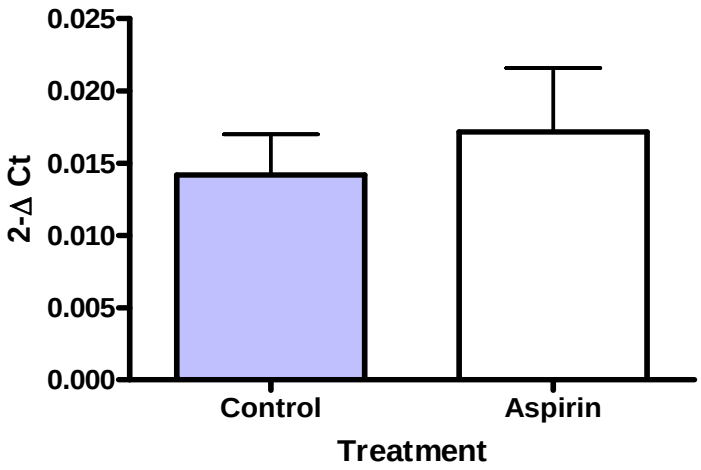
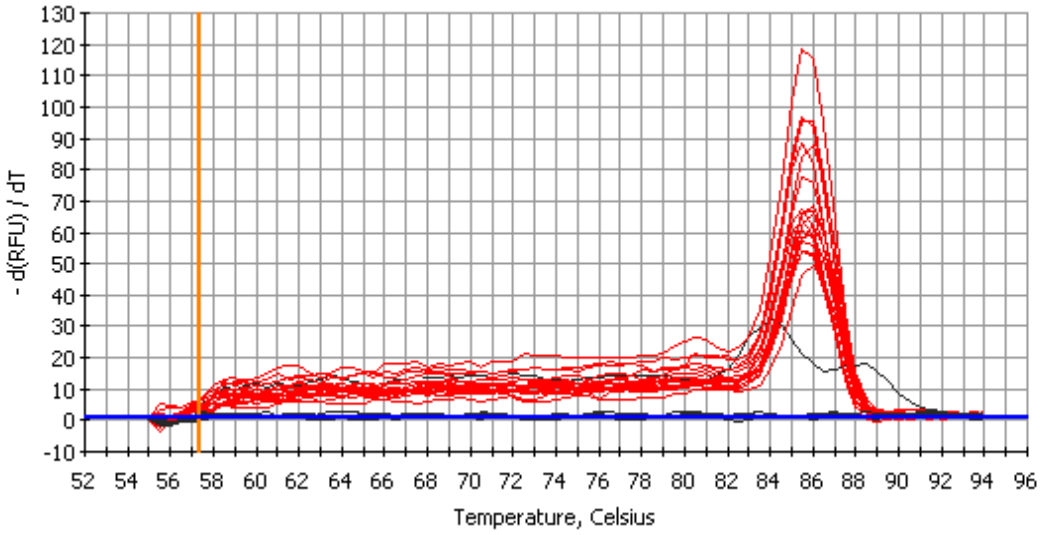


Figure 4.22 Amplification plot and melt curve analysis of *BRCA1* primer. (A) The melt curve shows a single peak indicating primer specificity. **(B)** Untreated and aspirin treated samples were run in triplicate in individual wells as indicated by the red traces and the black traces indicate negative control reactions. Each experiment was carried out 3 times and a representative image is shown.

(A)



(B)

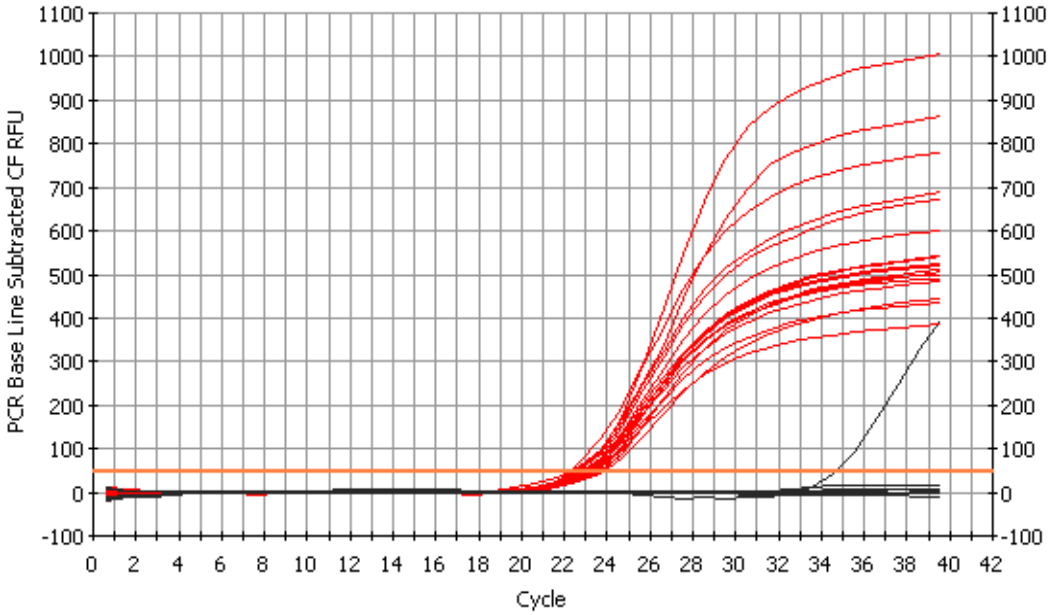


Figure 4.23. 48 hour incubation with 1 mM aspirin caused a significant increase in *XRCC3* mRNA expression compared to control.

The graph displays the relative expression of *XRCC3* to reference genes as calculated by comparison of Ct values (Δ – Δ Ct). Significant difference in relative expression was measured using an unpaired t-test. Results are displayed as means ± S.E.M. from 3 experiments performed in triplicate. $P < 0.05$ as indicated by * ($P = 0.0004$)

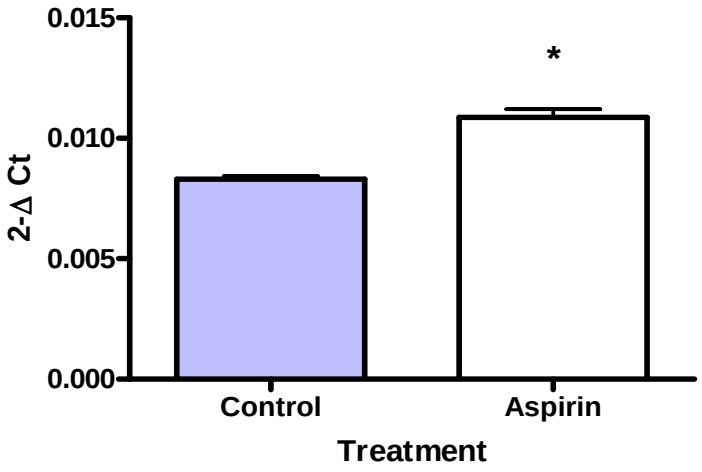
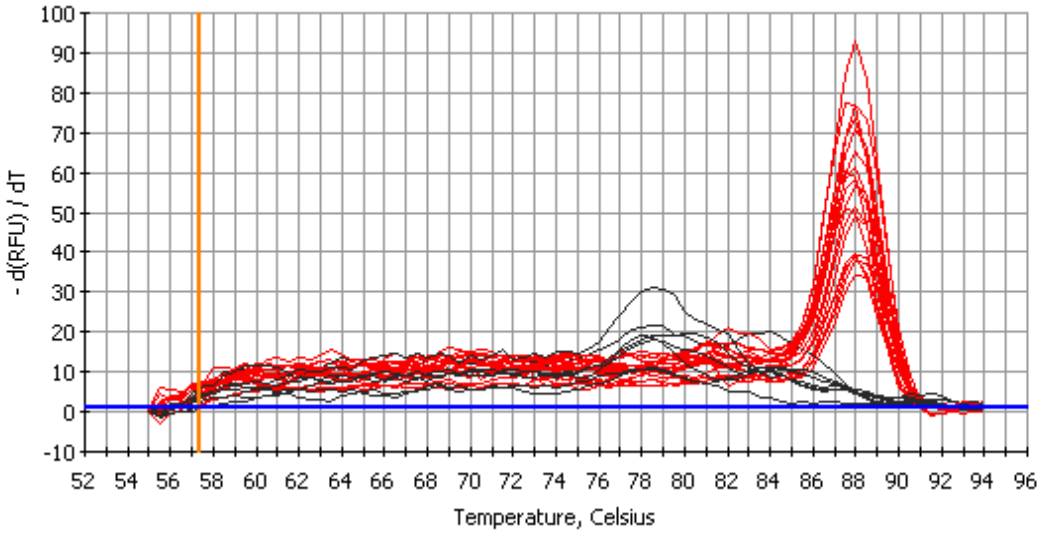


Figure 4.24. Amplification plot and melt curve analysis of *XRCC3* primer. (A) The melt curve shows a single peak indicating primer specificity. **(B)** Untreated and aspirin treated samples were run in triplicate in individual wells as indicated by the red traces and the black traces indicate negative control reactions. Each experiment was carried out 3 times and a representative image is shown.

(A)



(B)

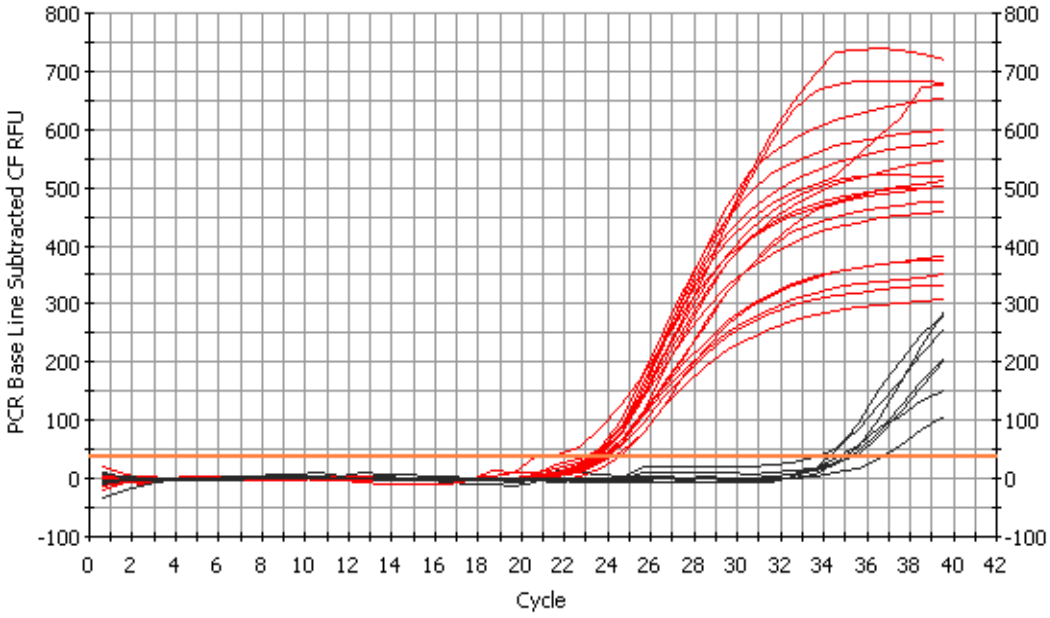


Figure 4.25. 48 hour incubation with 1 mM aspirin caused a significant increase in *GADD45α* mRNA expression compared to control.

The graph displays the relative expression of *GADD45α* to reference genes as calculated by comparison of Ct values (Δ – Δ Ct). Significant difference in relative expression was measured using an unpaired t-test. Results are displayed as means ± S.E.M. from 3 experiments performed in triplicate. $P < 0.05$ as indicated by * ($P = 0.0179$)

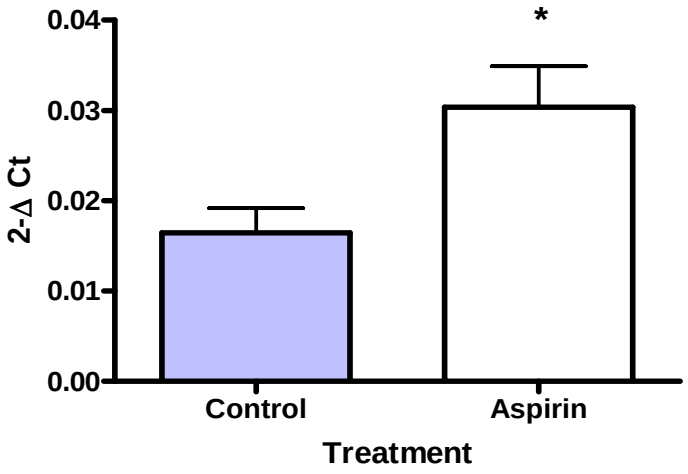
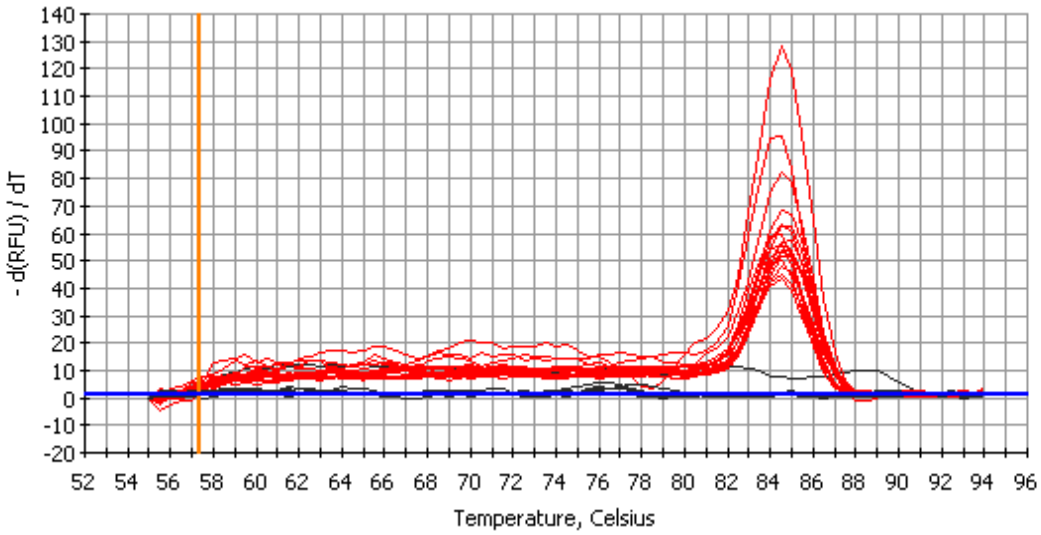
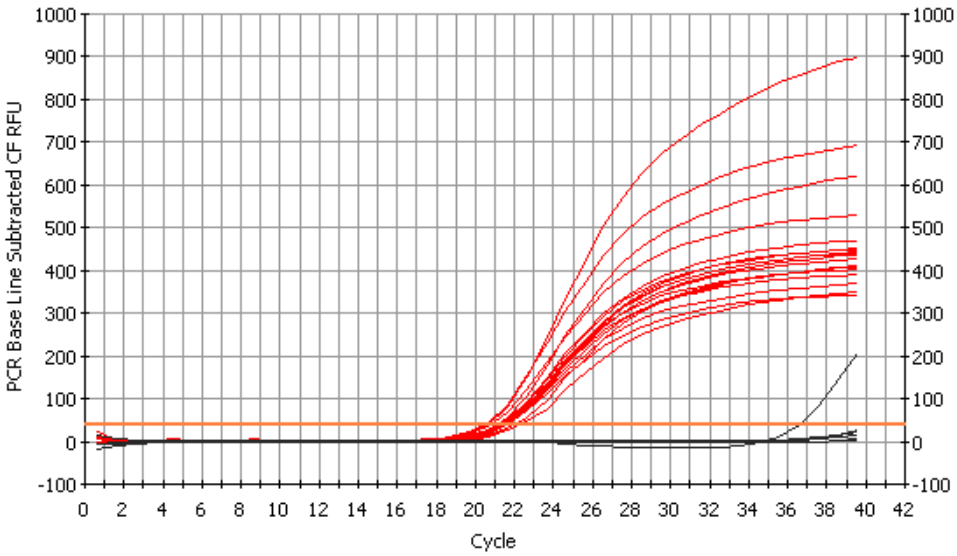


Figure 4.26. Amplification plot and melt curve analysis of *GADD45α* primer. (A) The melt curve shows a single peak indicating primer specificity. **(B)** Untreated and aspirin treated samples were run in triplicate in individual wells as indicated by the red traces and the black traces indicate negative control reactions. Each experiment was carried out 3 times and a representative image is shown.

(A)



(B)



4.6 Discussion

4.6.1 Cell Viability

The effects of aspirin and salicylic acid on cell viability of a panel of cancer cell lines including colon cancer (SW480, HCT116), breast cancer (MCF7, MDA231-MB) and glioma cells (U373MG) were investigated in this study. Figures 4.7 – 4.9 showed that in this panel of cell lines, aspirin was more cytotoxic to colon cancer cells than other cancer cell types with the exception of MCF7 cells. In contrast to research by Din *et al.,* (2004) the results in this present study showed that treatment with 5 mM and 10 mM aspirin caused a significant decrease in cell viability of MCF7 cells after 24 hour treatment (Figure 4.7). The results also showed a significant decrease in MCF7 cell viability after 48 hour treatment with 5 mM and 10 mM aspirin (Figure 4.8) and 72 hour treatment of MCF7 cells with 10 mM aspirin treatment (Figure 4.9) showing the most significant decrease in cell viability compared to the other cancer cell types. Aspirin cytotoxicity to MCF7 cells has previously been reported by Sali and Jewell (2006).

Interestingly, none of the cell lines in the panel except SW480 were affected at low concentrations, 0.5 and 1 mM of aspirin even after 72 hour treatment (Figure 4.9). SW480 sensitivity to aspirin compared to other colorectal cancer cell lines has previously been reported (Akashi *et al.,* 2000). In this study, HCT116 cells were less sensitive to aspirin treatment (Figure 4.7, Figure 4.8 and Figure 4.9) than SW480 cells (Figure 4.1, Figure 4.2 and Figure 4.3). Key differences between these cell lines are MMR status and p53 status. Research by Luciani *et al.,* (2007) showed by using HCT116 cells and also p53-null HCT116$^{p53-/-}$ that absence of p53 causes cells to become more resistant to aspirin treatment than cells which have wild type p53 suggesting a key role for p53 in changes to cell proliferation upon aspirin treatment. This is also suggested in studies by Din *et al* (2005). The present results would support this, both of the p53 mutant cell lines used in our panel (MDA231-MB and U373MG) are more resistant to aspirin than the p53 wild type cell lines (HCT116 and MCF7) used.

However, the results of this present study also suggest that the MMR status of the cell lines may also play a role. SW480 is a p53 mutant cell line yet results from this study show that it is highly sensitive to aspirin treatment even at low concentrations. In addition it is also shown that MCF7 cells have a higher sensitivity to aspirin than the other cell lines used in our panel. Both these cell lines are MMR proficient and this may account for their increased sensitivity to aspirin.

It is also interesting to note that results presented (Figure 4.1 – 4.6) show that a difference in cell viability effects of aspirin and salicylic acid was seen depending on diluent used and that the diluents themselves had effects on cell viability. One hundred percent ethanol affected cell viability more than Tris.HCl buffer (pH 7.2) which highlights the importance of selecting an appropriate diluent as the diluents themselves may cause effects and skew results and subsequent analysis.

4.6.2 MBD4 and Salicylates

An aim of this study was to analyse changes in *MBD4* gene expression upon aspirin treatment. Although the balance of results presented in this chapter (e.g., Figures 4.13 and 4.16A) suggest that aspirin does not increase *MBD4* expression, quantitative real-time PCR analysis determined that treatment with 1 mM salicylic acid for 48 hours did cause a statistically significant increase in *MBD4* gene transcription in SW480 cells compared to control cells (treated with a equivalent amount of Tris.HCl buffer, pH 7.2). It is important to note that selection of reference genes was key to this conclusion and should be taken into consideration when analysing previous research. Evidence of increased *MBD4* mRNA expression upon treatment with salicylic acid is presented in Figure 4.16B. This supports the hypothesis that *MBD4* is induced upon salicylate treatment. The induction of *MBD4* upon salicylic acid rather than aspirin treatment could indicate the significance of the acetyl group of aspirin. It could be considered that the acetyl group makes aspirin a more potent drug than salicylic acid. However, the transcriptional expression results suggest that salicylic acid may play a key role in initiating cell death as is it able to induce the expression of MBD4, a protein with known association with the MMR system in particular MLH1.

Two possible mechanisms could therefore be suggested for a pathway in which aspirin is able to initiate cell death. It could be suggested that it is the removal of the acetyl group from aspirin which triggers apoptotic mechanisms resulting in total cell death. The second pathway may be that the removal of the acetyl group would convert aspirin to salicylic acid and it is actually the salicylic acid which is responsible for initiating cell death. Salicylic acid may induce MBD4 (Figure 4.16B), which may in turn trigger apoptosis via a MMR system associated pathway. This may explain why in the cell viability assays, at higher concentrations aspirin treatment is able to demonstrate total cell death as there would be increased conversion of aspirin to salicylic acid and as a result an increase in salicylic acid and therefore the two pathways would both be induced to greater levels.

The hypothesis that the conversion of aspirin to salicylic acid may play a key role in triggering apoptosis could be further investigated by demonstrating an increased level of acetylases in the SW480 cell line compared to other cell lines, i.e., U373MG and MDA231-MB where aspirin and salicylic acid was not as effective in inducing cell death. A higher level of acetylases found would indicate that aspirin is more effective in inducing apoptosis within this cell line as there is a greater level of acetylases to convert the aspirin to salicylic acid. Further evidence of increased aspirin action on cancer cells in relation to levels of acetylases would also prove to be useful in determining this theory. Inhibition of acetylases in SW480 cells should block the conversion of aspirin to salicylic acid and therefore reduce or inhibit cell death, whereas inducing acetylases in cell lines found to be deficient or contain low levels (e.g. U373MG and MCF7) should increase conversion of aspirin to salicylic acid and should provide evidence of cell death which was not present before.

If it is indeed salicylic acid that is the essential factor for initiating cell death then research needs to confirm the metabolism of aspirin in the gut and whether the aspirin is actually converted to salicylic acid if correlations could be determined between this conversion in the gut and increased expression of MBD4 and whether there is any evidence for this mechanism of action to play a key role in to reducing the risk of colon cancer. It has been previously demonstrated by epidemiological studies and data that regular use of low dose aspirin based medication can reduce the risk of colorectal cancer, i.e., the use of aspirin is inversely associated with the risk of colorectal cancer (Rosenberg *et al.,* 1991; Thun *et al.,* 1991; Muscat *et al.,* 1994). It may be that the aspirin is actually being metabolised by the gut to salicylic acid and it is this salicylic acid which is initiating cell death and preventing colorectal cancer.

It has been reported that MBD4 is transcriptionally coupled with MLH1 (Bellacosa *et al.,* 1999) and any importance of this pairing in apoptotic mechanisms also needs to be elucidated further. The increased expression of MBD4 upon treatment with salicylic acid may be subsequently inducing the expression of MLH1 which may lead to further mechanisms for cell death. MLH1 is known to induce apoptosis when triggered by DNA damage (Hardman *et al.,* 2001). To further investigate the role between MLH1 and MBD4 it may be important to demonstrate changes in MLH1 expression with salicylic acid treatment and to determine whether these changes correlate with changes seen in MBD4 expression. Previous research has shown already demonstrated that expression of MLH1 increases after treatment with 1 mM aspirin for 48 hours (Goel *et al.,* 2003) however, salicylic acid effects on MLH1 expression have yet to be determined.

These results may indicate a pathway where aspirin is converted to salicylic acid, which induces MBD4 expression, which in turn increases apoptosis by FADD and increases MLH1 which in turn also induces apoptosis via other mechanisms. The subsequent increase in MBD4 expression shown with salicylic acid treatment may demonstrate that there is an induction in the FADD mechanism or even possibly a novel mechanism yet to be elucidated.

4.6.3 Aspirin and DNA repair signalling genes

This study also investigated the relationship between aspirin and DNA repair and signalling genes, utilising a commercially available RT^2 Profiler^{TM} PCR Array to analyse the expression of 84 different genes involved in DNA damage signalling pathways in the colorectal cancer cell line SW480 upon aspirin treatment. Microarray analysis has been extensively utilised to examine alterations in gene expression in response to NSAID exposure in a number of colon cancer cell lines with a subsequent large list of alterations in gene expression reported (Hardwick *et al.,* 2004; Huang *et al.,* 2006; Iizaka *et al.,* 2002; Germann *et al.,* 2003 and Yin *et al.,* 2006). However, this is the first study to utilise a RT^2 Profiler^{TM} PCR Array to assess the expression profile of a panel of genes involved in DNA damage signalling pathways upon aspirin treatment. When SW480 cells are exposed to 1 mM aspirin for 48 hours, a significant decrease in the expression of *BRCA1*, *ATR* and *MAPK12* and a significant increase in the expression of *XRCC3* occurs. However, validation of the results obtained from the RT^2 Profiler^{TM} PCR Array by quantitative real-time PCR was met with mixed success and highlights the importance of array validation.

In contrast to Goel *et al.,* (2003) who showed an up-regulation of protein expression of MLH1, MSH2, MSH6 and PMS2, the results in this study show no significant change in gene transcription of *MLH1*, *MSH2* or *PMS2* but did provide evidence that aspirin could affect the expression of several other key genes involved in DNA damage signalling pathways. In Figure 4.18, out of the 84 genes on the array, 27 genes were up-regulated and 57 genes were down-regulated (whether statistically significant or not) giving an overall impression of a down-regulation of DNA repair and damage signalling genes.

4.6.4 ATR

RT^2 Profiler™ PCR Array analysis showed a significant (P = 0.0014) 4.12 fold decrease in *ATR* mRNA expression in SW480 cells after 1 mM aspirin treatment for 48 hours compared to control. ATR belongs to the phosphoinositide 3-kinase related protein kinases (PIKKs) family alongside ATM and is responsible for regulating the DNA damage response, cell cycle progression and DNA recombination and as such a disruption in the ATR pathway causes genomic instability (Cimprich and Cortez, 2008). This is highlighted by Seckel syndrome, a known disorder caused by ATR gene mutation and is characterised by intrauterine growth retardation, dwarfism, microcephaly and mental retardation (O'Driscoll *et al.,* 2003). ATR is considered essential for viability of mouse and human cells as demonstrated in studies using ATR knockout mice models where lack of ATR proved lethal indicating its essential role in embryonic development (Brown and Baltimore, 2000). Also deletion of ATR in human cells ultimately led to cell death following loss of DNA damage checkpoint response (Cortez *et al.,* 2001). This could suggest that aspirin down-regulation of ATR may play a role in the decrease in cell viability of SW480 cells. ATR is involved primarily in the cell cycle checkpoint pathway. It is linked with a protein co-factor ATRIP (ATR-interacting protein) (Cortez *et al.,* 2001). Essentially the ATR-ATRIP complex is thought to sense single stranded DNA for example at stalled replication forks and also at points of double strand breaks caused by DNA damage. The activation of the complex is thought to be recruited by RPA (replication protein A) which binds to single stranded DNA (Zou and Elledge, 2003). The ATR-ATRIP complex co-localises with another complex, the RAD9-RAD1-HUS1 (9-1-1) complex. This 9-1-1 complex is structurally similar to PCNA (Proliferating cell nuclear antigen) - a heterotrimeric ring-shaped complex (Parrilla-Castellar *et al.,* 2004). One of the primary substrates ATR activates is Chk1 (Checkpoint kinase 1). Once phosphorylated Chk1 in turn phosphorylates Cdc25A leading to subsequent degradation of Cdc25A which regulates DNA replication. If ATR or Chk1 are down-regulated, an accumulation of Cdc25A occurs leading to an increase in double strand breaks and subsequent cell cycle arrest (Syljuasen *et al.,* 2005; Sorensen *et al.,* 2005). Other genes on the array associated with ATR also show a down-regulation in gene expression, including *RPA1, RAD9, HUS1* and *Chk1*. *RAD1* shows an up-regulation of gene transcription. However, these changes were found to be statistically non-significant.

Luciani *et al.,* (2007) have shown that aspirin blocks proliferation of colon cancer cells by inducing a G1 arrest through the activation of the ATM pathway. One reason to explain why a different response in ATM is shown in the present work, may be because of the lower concentration of

aspirin used in this study. It has been shown by other studies that treatments with different concentrations of aspirin give different gene expression patterns (Hardwick *et al.,* 2004; Yin *et al.,* 2006).

The idea that the cell cycle pathway is disrupted by aspirin has been previously suggested. Other studies have previously suggested cell cycle arrest to be responsible for the anti-proliferative effects of aspirin (Ricchi *et al.,* 1997). Decreased transcription of CCNB1 (Cyclin B1) which regulates cell-cycle progression at the G_2/M phase by sulindac treatment has also been reported (Iizaka *et al.,* 2002). In addition to this finding another study shows that aspirin caused a concentration dependent decrease of cell proportion at G_2/M phase (Lai *et al.,* 2008) and Goel *et al.,* (2003) also made a similar observation in their study.

4.6.5 BRCA1

Another known substrate of ATR is BRCA1 (Cortez *et al.,* 1999; Tibbets *et al.,* 2000). RT^2 Profiler™ PCR Array analysis showed a significant (P = 0.04) 6.59 fold decrease in *BRCA1* mRNA expression in SW480 cells after 1 mM aspirin treatment for 48 hours compared to control. The *BRCA1* gene was discovered in 1994 (Miki *et al.,* 1994) and is a known tumour suppressor with germline mutations causing breast and ovarian cancer susceptibility. The role of BRCA1 in DNA repair became apparent when it was found to co-localise with RAD51 (Scully et al., 1997a) suggesting it may be involved in maintaining genomic stability which was supported by further studies (Moynahan *et al.,* 1999). Therefore, as with a loss of ATR function, a loss of BRCA1 function may also increase genomic instability. BRCA1 disruption in mouse models caused embryonic lethality (Hakem *et al.,* 1996; Gowen *et al.,* 1996). Interestingly, BRCA1 deficient cells show an absence of G2/M checkpoint response to ionising radiation (Xu *et al.,* 2001). BRCA1 also interacts with the RAD50-MRE11-NBS1 complex involved in homologous recombination, non-homologous end joining, meiotic recombination and telomere maintenance (Zhong *et al.,* 1999). Interestingly, the array results of this present study show an increase in RAD50 and NBS1 and a decrease in MRE11 transcription, although these changes were found to be statistically non-significant. The down-regulation of transcription of the tumour suppressor gene *BRCA1* upon aspirin treatment is of interest. Recently published data indicates that regular NSAID and aspirin intake is associated with an increase in breast cancer (Friss *et al.,* 2008) and as such it would be interesting to see if *BRCA1* gene and/or protein expression is down-regulated upon aspirin treatment in breast cancer cells.

4.6.6 GADD45α

RT^2 Profiler™ PCR Array analysis showed a large though statistically non significant (P = 0.16) 14.78 fold increase in *GADD45α* mRNA expression in SW480 cells after 1 mM aspirin treatment for 48 hours compared to control. GADD45α belongs to the growth arrest and DNA damage (GADD) family of proteins along with GADD45, GADD45β, GADD45λ and GADD153 and an over-expression of GADD proteins leads to growth inhibition and/or apoptosis (Yu *et al.,* 2003). The up-regulation of *GADD45α* upon aspirin treatment has been previously reported (Iizaka *et al.,* 2002; Yin *et al.,* 2006; Sarkar *et al.,* 2002); thus, the up-regulation of *GADD45α* expression in this study can be interpreted to act as a positive control validating the PCR array data. Levels of GADD45α alter dependent on cell cycle phase with high expression at G1 phase and low expression at S phase (Hollander and Fornace, 2002).

The up-regulation of GADD45α upon aspirin and also sulindac treatment has been previously reported (Iizaka *et al.,* 2002; Yin *et al.,* 2006; Sarkar *et al.,* 2002). However, the significance of this has not been fully investigated and so the large fold up-regulation of GADD45α mRNA upon aspirin treatment shown by PCR array in this study is of key interest. Recent research has shown evidence for GADD45α involvement in the basis of action of non-steroidal anti-inflammatory drugs (NSAIDS). Treatment with NSAIDs resulted in the up-regulation of the cytokine melanoma differentiation associated gene-7/Interleukin-24 (IL-24) in a number of different cancer cell types which led to an over-expression of GADD45α resulting in cell cycle arrest (Zerbini *et al.,* 2006). In addition, experiments utilising RNAi to knock down GADD45α expression, showed apoptosis to be prevented in cells when treated with NSAIDS (Zerbini *et al.,* 2006). Using microarray analysis in prostate cancer cell lines, Zerbini *et al.,* (2006) identified a role for IL-24 as a mediator for NSAIDS in inducing growth arrest and apoptosis.

Intriguingly, IL-24 has been reported to activate the p38 MAPK pathway leading to an over-expression of the GADD family of genes in human melanoma cells and resulting in subsequent cytotoxicity (Sarkar *et al.,* 2002). Thoms *et al.,* (2007) have shown activation of p38 MAPK pathway by aspirin in SW480 cells. However, this study showed that the transcript level of a p38 isomer *MAPK12* was down-regulated upon aspirin treatment. RT^2 Profiler™ PCR Array analysis showed a statistically significant (P = 0.05) 2.44 fold decrease in *MAPK12* mRNA expression in SW480 cells after 1 mM aspirin treatment for 48 hours compared to control. This suggests that the *GADD45* gene transcription induction is possibly IL-24 and p38 MAPK pathway independent in

this experimental model. Interestingly, a recent study has shown a link between GADD45 and MBD4 (Rai *et al.,* 2008). This study shows that over-expression of GADD45α promotes DNA demethylation and suggests a physical interaction between GADD45α and MBD4 in Zebrafish.

4.6.7 XRCC3

RT^2 Profiler™ PCR Array analysis showed a significant (P = 0.05) 7.37 fold increase in *XRCC3* mRNA expression in SW480 cells after 1 mM aspirin treatment for 48 hours compared to control. XRCC3 is a paralog of RAD51 along with RAD51B, RAD51C, RAD51D and XRCC2 (Yonetani *et al.,* 2005) and interacts with RAD51C (Liu *et al.,* 1998). It is involved in DNA double strand break repair via the homologous recombination pathway (Thacker and Zdzienicka, 2004) and as such a disruption in XRCC3 expression could cause genomic instability. RT^2 Profiler™ PCR Array analysis also showed an increase in RAD51 (although, statistically non-significant).

4.6.8 Conclusion

In conclusion, the results presented in this chapter demonstrate conflicting evidence that MBD4 gene expression is up-regulated by salicylic acid treatment and may play a role in the cytotoxic mechanism of aspirin. Via RT^2 Profiler™ PCR array analysis aspirin was demonstrated to cause decreases in expression of transcripts of *ATR*, *BRCA1* and *MAPK12* and increases in *GADD45α* and *XRCC3* expression. These aspirin induced changes may affect many DNA repair and signalling pathway genes which suggests that aspirin could greatly affect DNA stability. It was therefore essential to demonstrate that changes in transcript levels correlated with the encoded protein abundance.

Chapter 5

The Effects of Aspirin on the Expression of DNA Repair Proteins

5 The Effects of Aspirin on the Expression of DNA Repair Proteins

5.1 Introduction

The central dogma of Crick (1956) proposed the one-way flow of information from DNA to RNA to protein and effectively this lays the philosophical foundation for the genome projects. If the prediction of DNA sequence leads to protein product identification then is it reasonable to expect that array technology which assures transcript abundance can also be indicative of product abundance? However, there is no definitive correlation between gene and protein abundance (Gygi *et al.,* 1999). Indeed, the first groups to measure gene transcript abundance utilising array technology did not analyse the corresponding protein abundance (Wodicka *et al.,* 1997; Lashkari *et al.,* 1997). This still continues to be the case in studies carried out analysing gene expression where correlation of protein abundance is not considered and as such can be viewed as a likely error source within the literature.

5.1.1 RT^2 Profiler™ PCR Array Results

RT^2 Profiler™ PCR Array analysis of gene expression in colorectal cancer cell line SW480 upon aspirin treatment showed novel findings in changes of transcription of key genes involved in DNA damage signalling pathways (Chapter 4). Specifically, significant decreases in *ATR*, *BRCA1* and *MAPK12* gene transcription and also a significant increase in *XRCC3* transcription and a large fold increase in *GADD45α* mRNA were observed (Table 4.1). However, validation of these results by quantitative real-time PCR (Figures 4.19, 4.21, 4.23 and 4.25) met with mixed success. So to further ascertain the effects of aspirin on DNA repair signalling, investigations were undertaken to determine the effects of aspirin on the protein expression of those genes found to be altered upon aspirin exposure by RT^2 Profiler™ PCR Array analysis.

Protein expression of BRCA1, ATR, XRCC3 and GADD45α was investigated by immunofluorescence and immunoblotting to determine any correlation with changes of mRNA expression. These proteins were chosen for analysis based on *P* value significance ($P < 0.05$) and difference in expression level between control and treated samples being > 4-fold. Whilst the *P* value for *GADD45α* is greater than 0.05 ($P = 0.16$), the large fold change and previous experimental research (Iizaka *et al.,* 2002; Yin *et al.,* 2006; Sarkar *et al.,* 2002) indicating an up-regulation of

GADD45α upon aspirin treatment warranted further investigation of this protein. Also, although the *P* value for *MAPK12* was 0.05, the difference was less than 4 fold and therefore *MAPK12* was not chosen for further analysis. In addition to immunoblotting and immunofluorescence analysis of XRCC3, a novel immunohistochemical method using analytical digital photomicroscopy (ADP) was also utilised to give semi-quantitative values for comparison analysis.

Numerous studies have suggested that regular aspirin intake increases the risk of breast cancer (Friss *et al.,* 2008). The novel finding by RT^2 Profiler™ PCR Array analysis which shows that *BRCA1* expression is decreased in colorectal cancer cells upon aspirin exposure is therefore of interest. If there is also a decrease in transcription of this breast cancer suppressor gene and the encoded protein expression in breast cancer cells upon aspirin treatment this may suggest a possible link to how aspirin may cause an increased risk in breast cancer.

Many studies have now shown a lack of correlation between gene transcript and protein abundance (Gygi *et al.,* 1999; Lichtinghagen *et al.,* 2002). Also Birrell *et al.,* (2002) show that transcript induction of *S.cerevisiae* by DNA damage did not necessarily identify genes required for response. Taking this into consideration, analysis of protein abundance of the key genes found to be altered in expression upon aspirin treatment in Chapter 4 was considered essential for this study.

5.2 Chapter Aims

The effects of aspirin on the expression of the DNA repair proteins ATR, BRCA1, XRCC3 and GADD45α in cell line SW480 were investigated to correlate with changes in protein expression and gene transcription, as determined by RT^2 Profiler™ PCR Array (Chapter 4).

5.3 Methods

5.3.1 Protein Assay Techniques

Immunoblotting, immunofluorescence and immunohistochemistry methods were used for protein analysis.

5.3.1.1 Immunoblot analysis

For immunoblot analysis, protein was extracted from SW480 cells, untreated or treated with 1 mM aspirin for 48 hours and quantified as described in 'Materials and Methods' (Chapter 2, Sections 2.3.1 and 2.3.2). Briefly, for protein extraction, cells (untreated or treated with 1mM aspirin) were repeatedly washed with PBS, then for GADD45α and XRCC3 protein analysis, protein was extracted using protein extraction buffer (20 mM Tris/150 mM NaCl/1 mM EDTA/1% Triton X) on ice for 1 hour, and centrifuged at 13,000 rpm to remove cell fragments. Protein was quantified using Bio-Rad's DC Protein Assay as per manufacturer's instructions. Extracted cellular proteins were then dissolved in 2 x Laemmli sample buffer, and analysed by discontinuous 12% SDS-PAGE, transferred to membrane and probed for protein expression. For XRCC3 and GADD45α gels, resolved proteins were transferred onto nitrocellulose membrane (Bio-Rad) at 100v for 1 hour by wet-transfer by standard protocol (Bio-Rad Laboratories, UK). The nitrocellulose was blocked with 5% (w/v) non-fat milk/0.2% (v/v) Tween 20 in TBS for 60 minutes at room temperature and incubated with primary antibody overnight at 4 ^{o}C, prior to repeated rinsing with 0.2% (v/v) Tween 20 in TBS, then incubated with a secondary HRP conjugated antibody for 60 minutes at room temperature, repeatedly rinsed with 0.2% (v/v) Tween 20 in PBS and visualised using ECL reagent according to manufacturer's instructions with a STORM Phosphorimager (GE Healthcare UK Limited, Buckinghamshire, UK).

For BRCA1 and ATR protein analysis, samples were extracted using RIPA buffer [10% NP-40 (v/v)/ 10% Na-deoxycholate (v/v)/ 100 mM EDTA in Tris buffer pH 7.4 including protease inhibitor cocktail tablet (Roche, Hertfordshire, UK), phosphatase inhibitor cocktail 1, phosphatase inhibitor cocktail 2 and 200 mM PMSF]. Cell pellets were thawed on ice, re-suspended in 3 bed volumes of extraction buffer and incubated on ice for 20 minutes. Whole cell extracts were obtained by centrifuging samples at 13,000 rpm at 4 ^{o}C for 15 minutes and samples were quantified using BCA

Protein Assay kit (Pierce, Rockford, IL, USA). Protein samples were analysed using pre-cast 3 – 8% Tris-Acetate gels (Invitrogen, Paisley, UK). Resolved proteins were transferred onto PVDF membrane (which had been equilibrated in 100% methanol and then equilibrated in transfer buffer for 15 minutes prior to transfer) at 350 mA for 1 hour. Before blocking, PVDF membrane was re-hydrated in 100% methanol and washed briefly in PBS. The membrane was soaked in blocking buffer [4% (w/v) Marvel^{TM} in PBS] for 1 hour at room temperature and incubated with primary antibody (diluted in blocking buffer) overnight at 4 ^{o}C. The membrane was then washed 3 times in wash solution [0.1% (v/v) Tween 20 in PBS] for 5 minutes each time and incubated with secondary antibody [diluted in 5% (w/v) Marvel^{TM}/0.1% (v/v) Tween 20 in TBS] for 1 hour at room temperature. The membrane was again washed 3 times using wash solution for 5 minutes each time and then incubated with Western Lightning^{TM} Plus-ECL oxidising reagent plus, enhanced Luminol reagent plus (PerkinElmer Life Sciences). The membrane was then analysed using Intelligent Dark box, LAS-3000 (Fujifilm) and Image Reader, LAS-3000 with an exposure time of 20 minutes.

Immunoblot experiments were performed on three different protein extractions for each treatment (1 mM aspirin or untreated). Although samples had been quantified to ensure equal concentrations of samples were loaded for comparison studies (20 µg total protein), to make certain of equal band loading, samples were also probed for beta-tubulin protein expression using a monoclonal anti-beta tubulin antibody (Sigma).

Immunoblotting analysis to investigate changes to BRCA1 protein expression in SW480 cells (Figure 5.3) and MCF7 cells (Figure 5.5) was carried out at KuDOS Pharmaceuticals, Cambridge, UK.

5.3.1.2 Immunofluorescence analysis

For immunofluorescence analysis, briefly, cells were fixed using a methanol/acetone mixture, washed and blocked with 5% BSA (w/v)/0.2% (v/v) Tween 20 in PBS then repeatedly washed with 0.2% (v/v) Tween 20 in PBS, and incubated with primary antibody at room temperature for 60 minutes. Cells were again washed with 0.2% (v/v) Tween 20 in PBS prior to incubation with the secondary antibody and incubated for 60 minutes at room temperature. Cells were washed with 0.2% (v/v) Tween 20 in PBS, then transferred to a glass slide whereupon they were analysed using a Carl Zeiss LSM 510 Meta confocal microscope.

5.3.1.3 Immunohistochemistry and Analytical Digital Photomicroscopy (ADP)

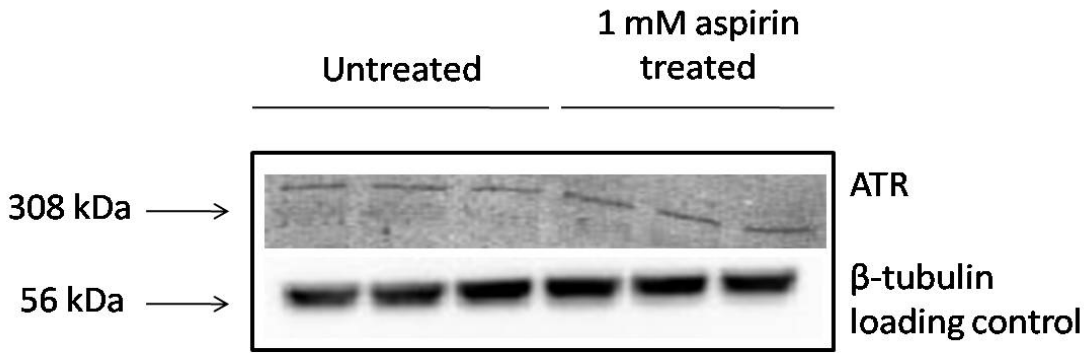
For immunohistochemical analysis, briefly, cells were fixed using a methanol/acetone mixture, washed and blocked with 5% BSA (w/v)/0.2% (v/v) Tween 20 in PBS then repeatedly washed with 0.2% (v/v) Tween 20 in PBS, and incubated with anti-XRCC3 antibody (1: 5000 dilution, Abcam, Cambridge, UK), at room temperature for 60 minutes. Cells were again washed with 0.2% (v/v) Tween 20 in PBS prior to incubation with the anti rabbit-HRP conjugated secondary antibody (1: 1000 dilution, Oncogene Research Products) and incubated for 60 minutes at room temperature. Cells were washed with 0.2% (v/v) Tween 20 in PBS, then incubated with 1-Step™ TMB substrate (Pierce, Rockford, IL, USA) for 4 minutes before the reaction was stopped with ultra-pure water. The coverslips were then mounted onto slides with mounting medium (Dako, CA, USA). Images were obtained using a Nikon Eclipse TS100 inverted microscope and Nikon DS-Fi1 camera head and subsequently analysed using analytical digital photomicroscopy (ADP) as outlined by Biocolor Ltd, UK (www.biocolor.co.uk). Briefly, the digitally acquired images were posterized using Adobe Photoshop software: blue pixels (as the substrate conversion resulted in a subtle blue stain) were counted in each image and recorded to give semi-quantitative values for comparison analysis. All photographs were taken at a constant magnification and all fields of view were of similar confluence. Three photographs were taken of each slide to obtain an average pixel value.

5.4 Analysis of ATR protein expression in colorectal cancer cell line SW480 following aspirin treatment

RT^2 Profiler™ PCR Array analysis showed a significant (P = 0.0014) 4.12 fold decrease in *ATR* mRNA expression in SW480 cells after 1 mM aspirin treatment for 48 hours compared to control (Table 4.2). In contrast quantitative real-time PCR validation analysis showed a slight, non-significant ($P > 0.05$) increase in mRNA expression (Figure 4.19).

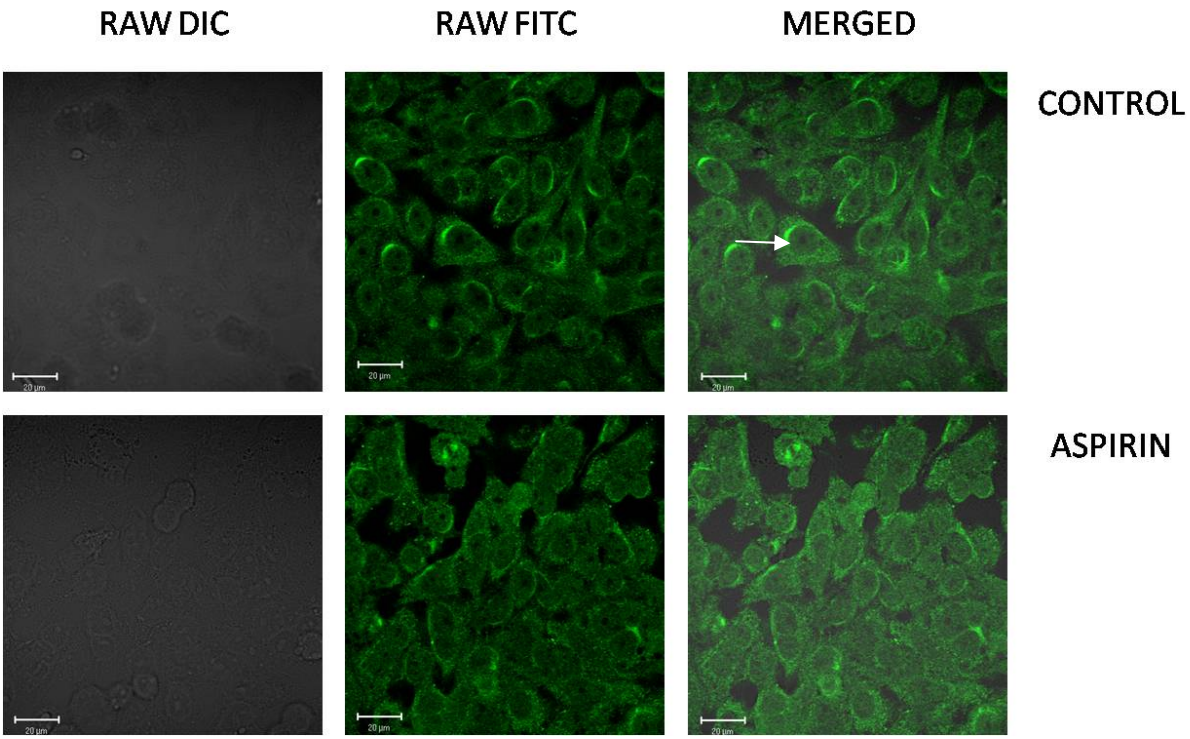
To investigate changes to ATR protein expression in SW480 cells, analysis was carried out using immunoblotting and immunofluorescence techniques as described in 'Materials and Methods' (Chapter 2, Section 2.3.5 and 2.3.9).

Figure 5.1 Immunoblot analysis of ATR expression in colorectal cancer cell line SW480 following aspirin treatment. Immunoblot analysis was carried out as described in 'Materials and Methods' (Chapter 2, Section 2.3.5). Membranes were probed with rabbit anti-ATR antibody (Cell Signalling) and anti-rabbit HRP antibody (Oncogene Research Products).



The results demonstrate no obvious change in expression of ATR protein upon treatment with 1 mM aspirin over a 48 hour period compared with untreated samples.

Figure 5.2 Immunofluorescence analysis of ATR expression in colorectal cancer cell line SW480 following aspirin treatment. Images were captured with an LSM 510 Meta confocal microscope using a x 63 objective and analysed with LSM 5 Meta software (Carl Zeiss IMT Corporation, Thornwood, NY). Bar, 20 µm. SW480 cells were probed with rabbit anti-ATR (Cell Signalling) and donkey anti-rabbit FITC (Santa Cruz). Images demonstrated no change in expression of ATR protein upon treatment with 1 mM aspirin over a 48 hour period compared with untreated samples. (RAW DIC = Raw Differential Interference contrast; RAW FITC = FITC channel only; MERGED = Merged RAW DIC and RAW FITC images).



Protein analysis of ATR expression by immunoblotting (Figure 5.1) and immunofluorescence (Figure 5.2) demonstrated that ATR protein expression in SW480 cells was not greatly affected by 48 hour treatment with 1 mM aspirin.

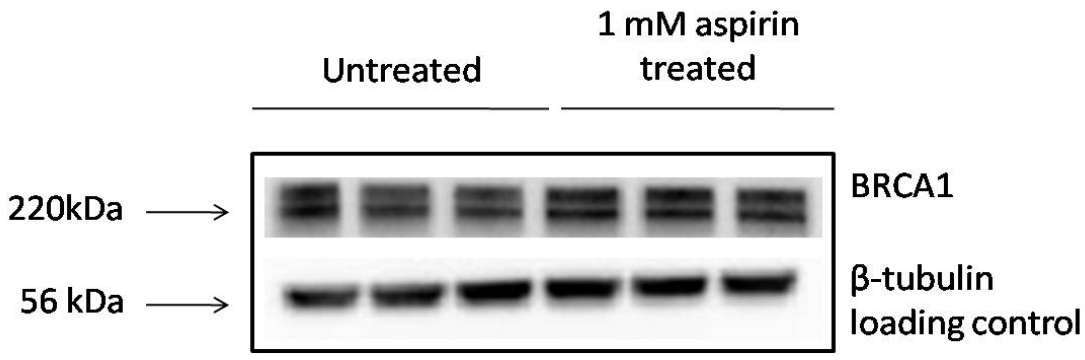
Interestingly, immunofluorescence analysis shows that in untreated cells there was a clear, distinct area within the nucleus where fluorescence did not occur (as indicated by arrow). However after aspirin treatment these areas became less distinct in some cells, indicating a possible re-localisation of the ATR protein.

5.5 Analysis of BRCA1 protein expression in colorectal cancer cell line SW480 following aspirin treatment

RT^2 Profiler™ PCR Array analysis showed a significant (P = 0.04) 6.59 fold decrease in BRCA1 mRNA expression in SW480 cells after 1 mM aspirin treatment for 48 hours compared to control (Table 4.2). In contrast quantitative real-time PCR validation analysis showed a small, non significant (P > 0.05) increase in mRNA expression (Figure 4.21).

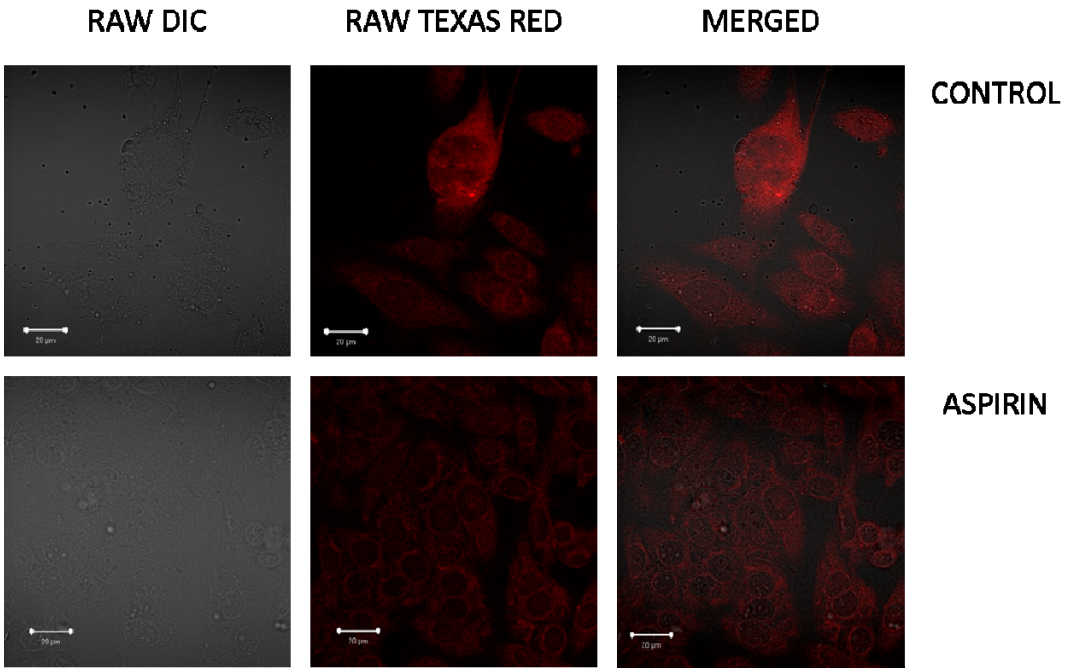
To investigate changes to BRCA1 protein expression in SW480 cells analysis, immunoblotting and immunofluorescence techniques were utilised as described in 'Materials and Methods' (Chapter 2, Sections 2.3.5 and 2.3.9). Immunoblotting analysis was carried out at KuDOS Pharmaceuticals, Cambridge, UK.

Figure 5.3 Immunoblot analysis of BRCA1 expression in colorectal cancer cell line SW480 following aspirin treatment. Immunoblot analysis was carried out as described in 'Materials and Methods' (Chapter 2, Section 2.3.5). Membranes were probed with mouse anti-BRCA1 antibody (Cell Signalling) and anti-mouse HRP antibody (Sigma).



The results demonstrated no obvious change in expression of BRCA1 protein upon treatment with 1 mM aspirin over a 48 hour period compared with untreated samples.

Figure 5.4 Immunofluorescence analysis of BRCA1 expression in colorectal cancer cell line SW480 following aspirin treatment. Images were captured with an LSM 510 Meta confocal microscope using a x 63 objective and analysed with LSM 5 Meta software (Carl Zeiss IMT Corporation, Thornwood, NY). Bar, 20 µm. SW480 cells were probed with mouse anti-BRCA1 (Cell Signalling) and donkey anti-mouse Texas-Red (Santa Cruz). Images demonstrated no change in expression of BRCA1 protein upon treatment with 1 mM aspirin over a 48 hour period compared with untreated samples. (RAW DIC = Raw Differential Interference contrast; RAW TEXAS RED = TEXAS RED channel only; MERGED = Merged RAW DIC and RAW TEXAS RED images).



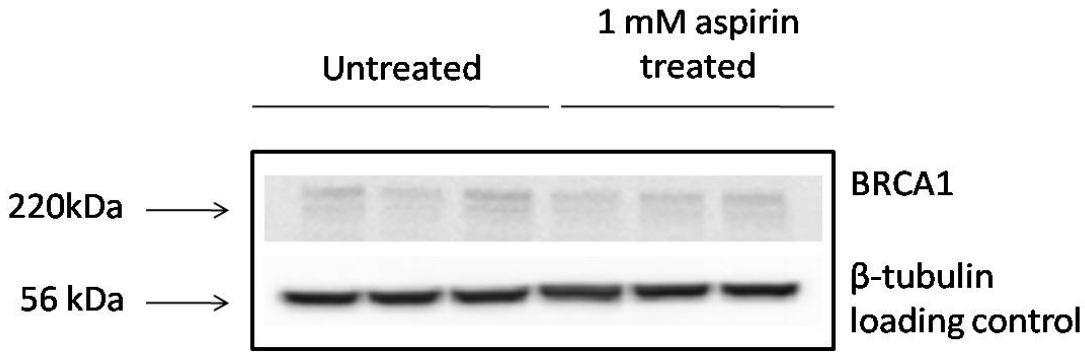
Figures 5.3 and 5.4 do not provide conclusive evidence that BRCA1 protein expression in SW480 cells is down-regulated by 1 mM aspirin treatment for 48 hours.

5.5.1 Analysis of BRCA1 protein expression in breast cancer cell line MCF7 following aspirin treatment

BRCA1 protein expression was examined in the breast cancer cell line MCF7 after treatment with 1 mM aspirin for 48 hours compared to untreated samples. MCF7 cells show sensitivity to aspirin treatment in a concentration-dependent and time-dependent manner (Figures 4.7, 4.8 and 4.9). Like SW480 cells, MCF7 cells are MMR proficient, however in contrast to SW480 cells they have a wild type p53 activity. Although Figure 4.8 showed that 1 mM aspirin treatment for 48 hours did not cause a significant decrease in MCF7 cell viability, this concentration was used as it is physiologically relevant rather than a higher concentration for example 5 mM or 10 mM which did cause significant decreases in cell viability.

To investigate changes to BRCA1 protein expression in MCF7 cells, immunoblotting and immunofluorescence techniques were utilised as described in 'Materials and Methods' (Chapter 2, Sections 2.3.5 and 2.3.9). Immunoblotting analysis was carried out at KuDOS Pharmaceuticals, Cambridge, UK.

Figure 5.5 Immunoblot analysis of BRCA1 expression in breast cancer cell line MCF7 following aspirin treatment. Immunoblot analysis was carried out as described in Materials and Methods' (Chapter 2, Section 2.3.5). Membranes were probed with mouse anti-BRCA1 antibody (Cell Signalling) and anti-mouse HRP antibody (Sigma).



The results demonstrated no detectable change in expression of BRCA1 protein upon treatment with 1 mM aspirin over a 48 hour period compared with untreated samples.

Figure 5.5 shows that BRCA1 protein expression is relatively weak in MCF7 cells as observed by faint bands compared to expression in SW480 cells (Figure 5.3). Again the results do not show conclusively whether BRCA1 expression is significantly altered although Figure 5.5 does show evidence of possible decrease in BRCA1 expression upon aspirin treatment as these bands appear to be less intense than the bands for untreated samples. Further experimentation was unable to be carried due to limited time available at KuDOS Pharmaceuticals.

5.6 Analysis of GADD45α protein expression in colorectal cancer cell line SW480 following aspirin treatment

RT^2 Profiler™ PCR Array analysis showed a statistically non-significant (P = 0.16) 14.78 fold increase in *GADD45α* mRNA expression in SW480 cells after 1 mM aspirin treatment for 48 hours compared to control (Table 4.2). However, in contrast, quantitative real-time PCR validation analysis showed a statistically significant ($P < 0.05$) increase in mRNA expression (Figure 4.25). This data together with previous research indicating an up-regulation of GADD45α upon aspirin treatment (Iizaka *et al.,* 2002; Yin *et al.,* 2006; Sarkar *et al.,* 2002) prompted an investigation into changes to GADD45α protein expression. Immunoblotting and immunofluorescence techniques were carried out as described in 'Materials and Methods' (Chapter 2, Sections 2.3.5 and 2.3.9).

Figure 5.6 Immunoblot analysis of GADD45α expression in colorectal cancer cell line SW480 following aspirin treatment. Immunoblot analysis was carried out as described in Materials and Methods' (Chapter 2, Section 2.3.5). Membranes were probed with rabbit anti-GADD45α antibody (Santa Cruz) and anti-rabbit HRP antibody (Oncogene Research Products).

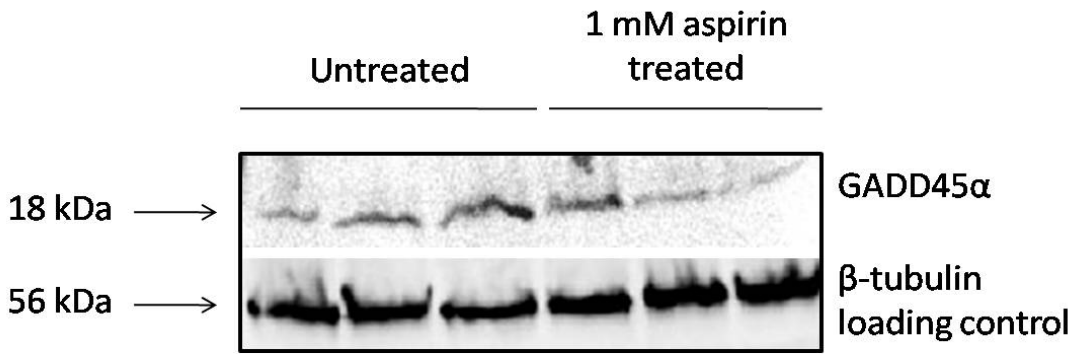
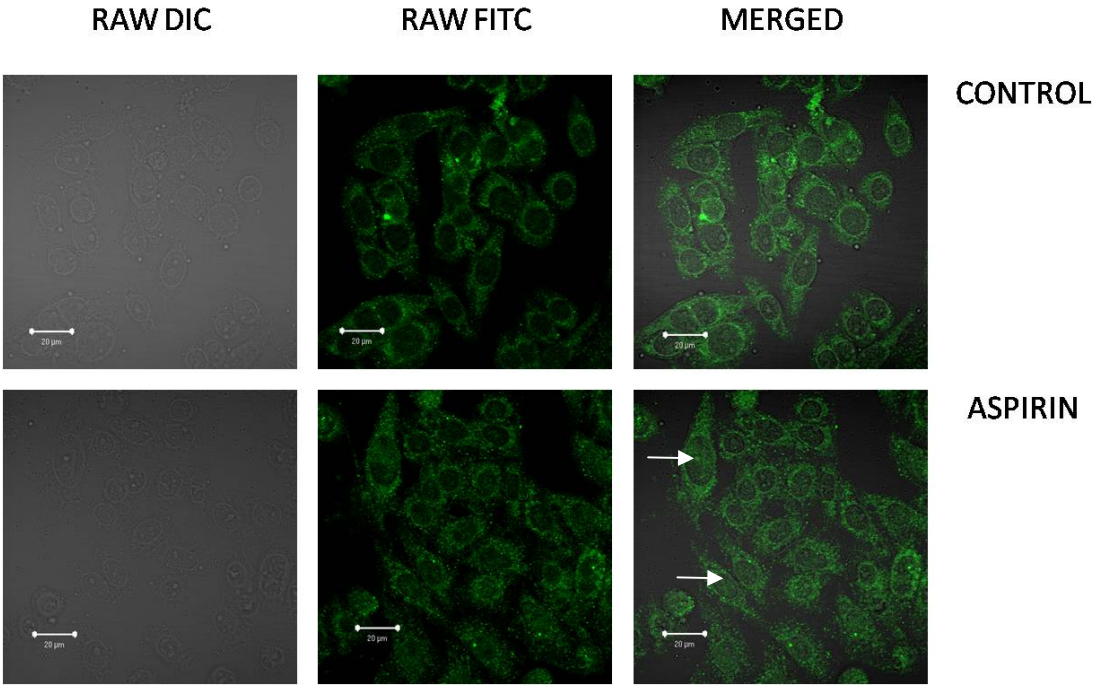


Figure 5.6 demonstrated no detectable change in expression of GADD45α protein upon treatment with 1 mM aspirin over a 48 hour period compared with untreated samples.

Figure 5.7 Immunofluorescence analysis of GADD45α expression in colorectal cancer cell line SW480 following aspirin treatment. Images were captured with an LSM 510 Meta confocal microscope using a x 63 objective and analysed with LSM 5 Meta software (Carl Zeiss IMT Corporation, Thornwood, NY). Bar, 20 µm. SW480 cells were probed with rabbit anti-GADD45α (Santa Cruz) and donkey anti-rabbit FITC (Santa Cruz). Images demonstrated no change in expression of GADD45α protein upon treatment with 1 mM aspirin over a 48 hour period compared with untreated samples. (RAW DIC = Raw Differential Interference contrast; RAW FITC = FITC channel only; MERGED = Merged RAW DIC and RAW FITC images).



Immunoblotting (Figure 5.6) indicated that GADD45α protein expression in SW480 cells was not obviously up-regulated by 1 mM aspirin treatment for 48 hours. However, immunofluorescence analysis (Figure 5.7) provides evidence for possible re-localisation of the protein within the cell. In 1 mM aspirin treated cells there appears to be an increase in fluorescence within the nucleus of the cell (as indicated by arrows) compared to untreated cells. This suggests that whilst detectable overall protein expression is not increased, a change to the protein distribution within the cells was occurring.

5.7 Analysis of XRCC3 protein expression in colorectal cancer cell line SW480 following aspirin treatment

RT^2 Profiler™ PCR Array analysis showed a significant (P = 0.05) 7.37 fold increase in *XRCC3* mRNA expression in SW480 cells after 1 mM aspirin treatment for 48 hours compared to control (Table 4.2). Validation of this result by quantitative real-time PCR analysis also showed a significant (P < 0.05) increase in mRNA expression (Figure 4.23).

To investigate changes to XRCC3 protein expression in SW480 cells analysis was carried out using immunoblotting, immunofluorescence and immunohistochemical techniques as described in 'Materials and Methods' (Chapter 2, Sections 2.3.5, 2.3.9 and 2.3.10). For immunohistochemical analysis analytical digital photomicroscopy (ADP) was used, as outlined by the Biocolor company and described in 'Materials and Methods' (Chapter 2, Section 2.5.2). In contrast to standard immunohistochemical analysis of the images to determine any change in XRCC3 protein expression levels which usually relies on independent observers to corroborate if changes in expression levels are seen. Analytical digital photomicroscopy (ADP) allows for a semi-quantitative analysis of protein expression levels.

Commercially available image analysis software (Adobe® Photoshop®) was utilised to calculate the blue pixels in each digitally acquired image. Blue pixels were calculated as in these experiments the substrate conversion results in a blue stain. The pixel value for each image was recorded and used to statistically determine changes in XRCC3 protein expression in SW480 cells upon 1 mM aspirin treatment for 48 hours.

Figure 5.8 Immunoblot analysis of XRCC3 expression in colorectal cancer cell line SW480 following aspirin treatment. Immunoblot analysis was carried out as described in Materials and Methods' (Chapter 2, section 2.3.5). Membranes were probed with rabbit anti-XRCC3 antibody (Abcam) and anti-rabbit HRP antibody (Oncogene Research Products).

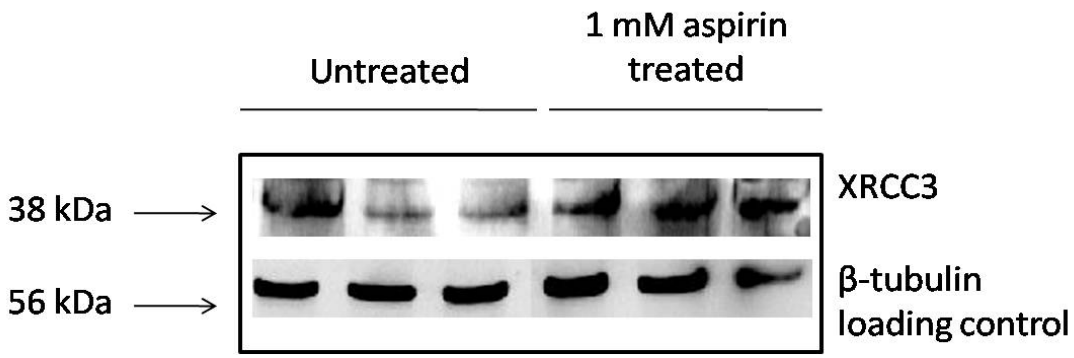


Figure 5.8 demonstrates an increase in expression of XRCC3 protein upon treatment with 1 mM aspirin over a 48 hour period compared with untreated samples.

Figure 5.9 Immunofluorescence analysis of XRCC3 expression in colorectal cancer cell line SW480 following aspirin treatment. Immunofluorescence was carried out as described in 'Materials and Methods' (Chapter 2, Section 2.3.9). Images were captured with an LSM 510 Meta confocal microscope and analysed with LSM 5 Meta software (Carl Zeiss IMT Corporation, Thornwood, NY). Bar, 20 µm. SW480 cells were probed with rabbit anti-XRCC3 (Abcam) and donkey anti-rabbit FITC (Santa Cruz). Images demonstrated an increase in expression of XRCC3 protein upon treatment with 1 mM aspirin over a 48 hour period compared with untreated samples. (RAW DIC = Raw Differential Interference contrast; RAW FITC = FITC channel only; MERGED = Merged RAW DIC and RAW FITC images).

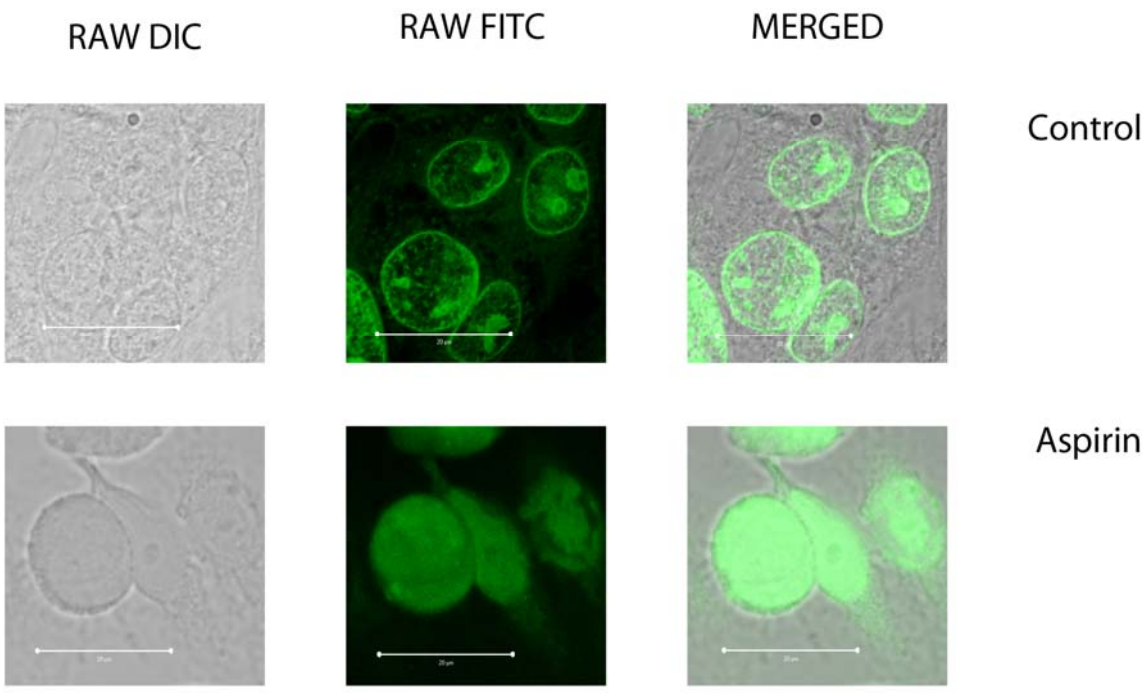
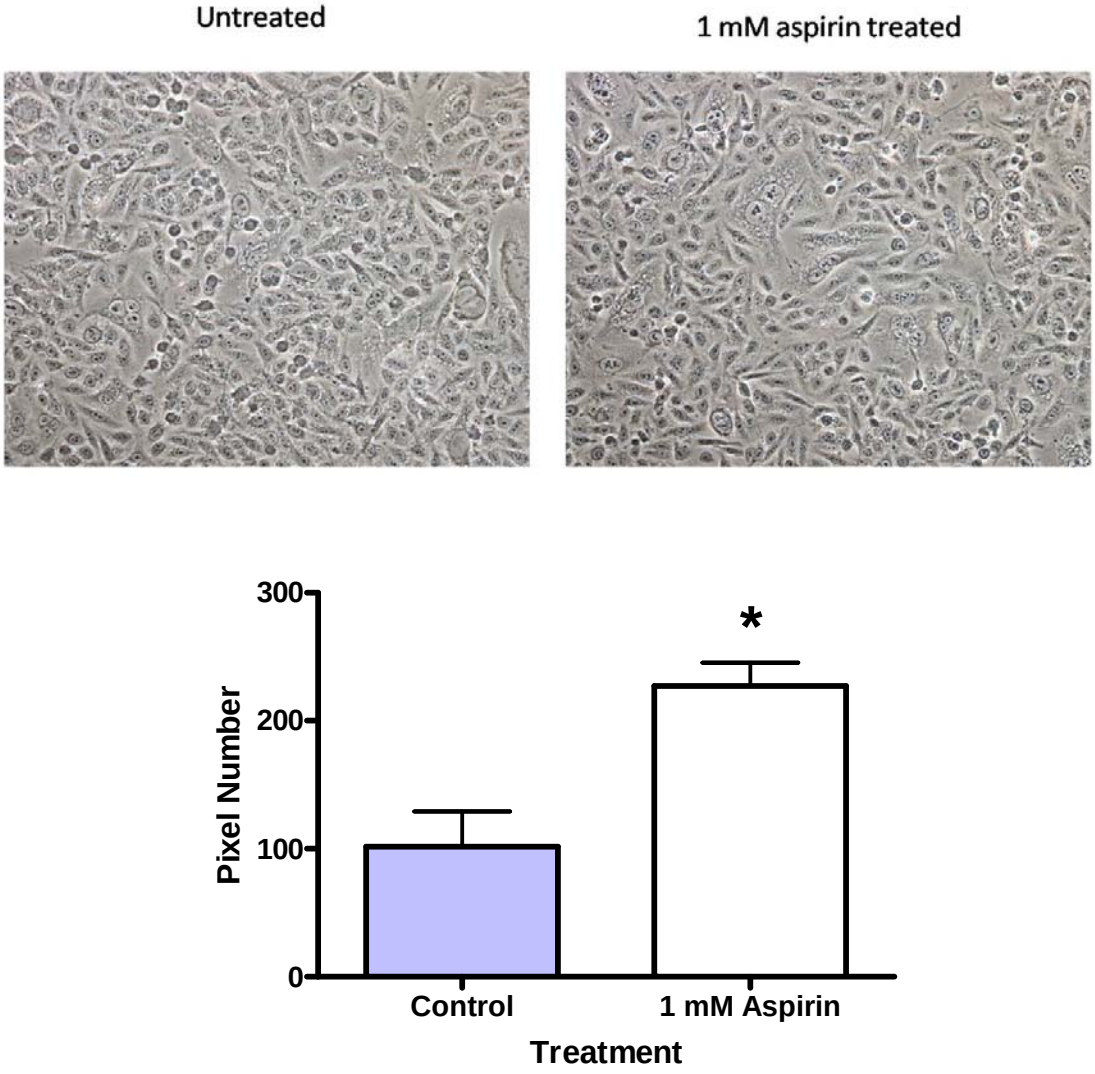


Figure 5.10 Immunohistochemical analysis of SW480 cells for XRCC3 expression. Digital images of SW480 cells untreated or treated with 1 mM aspirin for 48 hours were taken at x 200 magnification. To quantify the subtle alterations in substrate development, pixel values were quantified using analytical digital photomicroscopy as detailed in 'Materials and Methods' (Chapter 2, Section 2.5.2). Statistical analysis of pixel number indicated a significant increase ($P > 0.05$ as denoted by *, using unpaired t-test) in aspirin treated cells compared to untreated cells, showing an increase in XRCC3 protein expression upon aspirin treatment. Controls with secondary antibody only and primary antibody only were also carried out and pixel number analysis demonstrated values lower than those of experimental slides, indicating specificity of the reaction and that the antibodies on their own did not cause any significant change to pixel number (data not shown).



Results shown in Figure 5.8 and Figure 5.9 show that XRCC3 protein expression in SW480 cells was increased by 1 mM aspirin treatment for 48 hours compared to control samples. This correlates with changes seen in mRNA expression (Chapter 4). However, as altered protein expression is difficult to meaningfully quantify using these techniques, to further analyse protein expression, immunohistochemical analysis using ADP was also carried out.

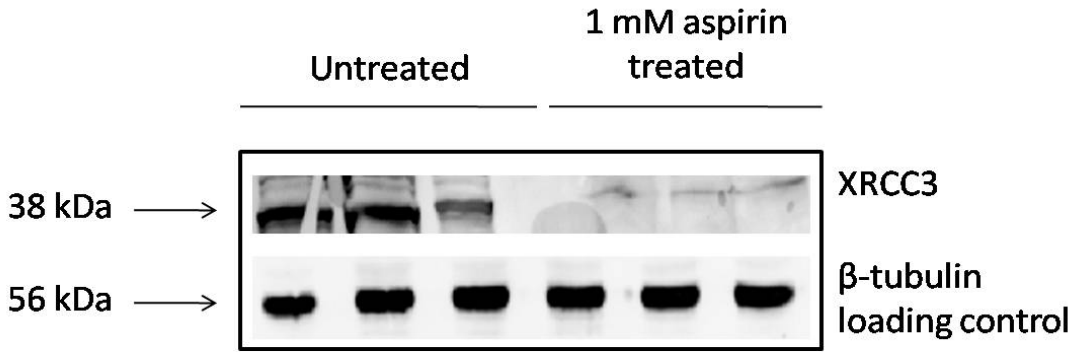
Figure 5.10 shows that an increase in blue pixels was observed in images taken of SW480 cells treated with 1 mM aspirin for 48 hours compared to images taken of untreated cells. This increase in pixel number directly relates to an increase in XRCC3 protein and as such the results correlate with the increase in XRCC3 protein expression observed in SW480 cells upon aspirin treatment by immunoblotting (Figure 5.8) and immunofluorescence (Figure 5.9).

5.8 Analysis of XRCC3 protein expression in colorectal cancer cell line HCT116 following aspirin treatment

To further investigate XRCC3 expression upon aspirin treatment in colorectal cancer, protein expression in the MMR deficient colorectal cancer cell line HCT116 was investigated. Unlike SW480 cells, the HCT116 cell line is MLH1 deficient and has wild type p53 activity. As shown in Chapter 4, HCT116 cells also showed a concentration –dependent and time-dependent decrease in cell viability upon aspirin treatment. However, the cells were not shown to be as sensitive to aspirin exposure as SW480 cells (Figures 4.7 , 4.8 and 4.9)

In contrast to XRCC3 expression in SW480 cells, 1 mM aspirin treatment for 48 hours caused a dramatic decrease in XRCC3 protein expression in HCT116 cells (Figure 5.11).

Figure 5.11 Immunoblot analysis of XRCC3 expression in colorectal cancer cell line HCT116 following aspirin treatment. Immunoblot analysis was carried out as described in Materials and Methods' (Chapter 2, Section 3.3.5). Membranes were probed with rabbit anti-XRCC3 antibody (Abcam) and anti-rabbit HRP antibody (Oncogene Research Products).



5.9 Discussion

5.9.1 Synopsis of Results

Gene expression profiling has in recent years become an increasingly fashionable and widespread methodology. However the information from such arrays is limited and the relationship between gene expression and the corresponding protein abundance needs to be explored to provide a better understanding of the mechanisms at a cellular level. The initial expectation for this part of the study was that aspirin might prove to have a role in regulating ATR, BRCA1, XRCC3 and GADD45α protein expression in colorectal cancer cells in correlation with its effects on *ATR*, *BRCA1*, *XRCC3* and *GADD45α* mRNA expression as observed by RT^2 Profiler™ PCR Array analysis in Chapter 4. However, the results fail to show any conclusive evidence that aspirin has any significant effect on alteration of protein levels of ATR (Figures 5.1 and 5.2), BRCA1 (Figures 5.3 and 5.4) and GADD45α (Figures 5.6 and 5.7) in correlation with its effects on corresponding mRNA expression. Nonetheless this study did find evidence for a strong correlation for *XRCC3* gene and protein expression. An increase in XRCC3 protein expression in SW480 cells upon 1 mM aspirin treatment for 48 hours (Figures 5.8, 5.9 and 5.10) in correlation with mRNA changes was observed. Further to this XRCC3 expression was analysed in the MMR deficient colorectal cancer cell line HCT116 and interestingly a decrease in XRCC3 expression upon 1 mM aspirin treatment for 48 hours was observed (Figure 5.11) suggesting that aspirin may dysregulate XRCC3 expression.

5.9.2 Correlation between transcript levels and protein abundance

The relationship between transcript levels and protein abundance is poorly understood. The correlation between mRNA and protein abundance has recently been reviewed by Maier *et al*., (2009). The authors point out that even though most research uses gene transcription data as evidence for protein expression, this is not always valid and research investigating correlation between mRNA and protein is weakly reported both in bacterial and mammalian cells. Although some research has shown a correlation between transcript and protein levels (Celis *et al.,* 2000; Anderson and Seilhamer, 1997; Orntoft *et al.,* 2002; Futcher *et al.,* 1999), a significant number of studies show a distinct lack of correlation between mRNA expression and protein abundance (Chen *et al.,* 2002; Conrads *et al.,* 2005; Lichtinghagen *et al.,* 2002; Gygi *et al.,* 1999; Lee *et al.,* 2003; Pascal *et al.,* 2008; Goel *et al.,* 2003). Interestingly, Goel *et al.* (2003) reported an up-

regulation in the expression of the DNA mismatch repair proteins MLH1, MSH2, MSH6 and PMS2 in a colorectal cancer cell line exposed to aspirin whilst showing no significant alteration in transcription assessed by quantitative multiplex RT-PCR, again clearly suggesting that transcript levels and protein abundance do not necessarily correlate. It should also be pointed out that other microarray studies investigating the effects of aspirin on colorectal cancer have not looked at the changes in corresponding protein levels (Iizaka *et al.,* 2002; Germann *et al.,* 2003; Yin *et al.,* 2006).

The lack of correlation between mRNA and protein levels may be a result of multiple factors and there are three suggested possible reasons to explain the poor correlation observed; (1) post-transcriptional parameters; (2) post translational parameters and (3) noise and experimental error, although a combination of these factors may together affect correlation (Greenbaum *et al.,* 2003; Maier *et al.,* 2009). Factors such as transcriptional splicing, post-transcriptional splicing, translational modifications, translational regulation and protein complex formation may affect mRNA and protein correlation. Translation efficiency has been shown to affect mRNA and protein correlation in bacteria, specifically features at elongation stages, i.e., codon usage and amino acid composition (Nie *et al.,* 2006). It may also be that technical methodologies are not highly accurate in quantification of mRNA or protein levels and these discrepancies may account for lack of correlation observed.

Post transcriptional mechanisms mean that there is not always a direct correlation between mRNA expression and the corresponding protein abundance. It is possible that the cells are able to compensate at the protein level for the possible transcriptional defects that are mediated by aspirin treatment, especially if the transcriptional changes are not very prominent and rather subtle. This may reflect alterations in mRNA stability or translation in addition to the stability of the protein product or altered subcellular locations, for example as shown by NFκB (Watson, 2006; Stark *et al.,* 2001).

Chen *et al.,* (2002) investigated the correlation between mRNA and protein levels in lung adenocarcinomas and found that only a subset of proteins showed significant correlation with mRNA levels. They suggest that protein isoforms need to be taken into consideration and show that the majority of separate isoforms did not correlate with mRNA levels, indicating that separate isoforms of the same gene product may be regulated differently (Chen *et al.,* 2002). Interestingly, they also observed examples of protein expression with a negative correlation to

mRNA expression, which has also been observed by others (Guo *et al.,* 2008) suggesting that there might be a negative feedback mechanism which is currently not understood (Chen *et al.,* 2002).

One other key factor which may influence mRNA and protein correlation is protein abundance. Research which reports a good correlation between mRNA and protein abundance may be influenced by the choice of protein product, for example the study may only have considered one or a few proteins to determine correlation between mRNA and protein abundance and those proteins chosen are well resolved and abundant within the cell (Gygi *et al.,* 1999; Celis *et al.,* 2000). Another aspect related to protein abundance is time delay. A recent study by Fournier *et al*., (2009) demonstrates that time can influence mRNA and protein correlation. The study analysing the effects of rapamycin in yeast shows that there was a time delay between mRNA and protein correlation, i.e., protein abundance at 6 hours correlated with mRNA expression at 2 hours. This may be due to the time needed for protein to accumulate or degrade. It also suggests in this case that the effects of rapamycin may not be immediate after addition of the drug, which is a point for consideration for this present study. It may be that transcriptional changes observed in SW480 cells upon aspirin treatment may not correlate with changes in protein expression because of this time delay affecting protein abundance. Further to this, post translational mechanisms affecting protein half lives also need to be taken into consideration.

There are also suggestions that sub-cellular localisation and function of protein may influence mRNA and protein correlation (Greenbaum *et al.,* 2003; Guo *et al.,* 2008). Greenbaum *et al.,* (2003) show that localisation of protein could affect mRNA and protein correlation, for example, they show that proteins in the nucleolus have a high correlation with mRNA levels and mitochondrial proteins have weak correlation. Findings by Guo *et al.,* (2008) suggest that proteins with essential molecular function such as protein binding, enzyme activity and signal transduction have a high correlation with mRNA levels possibly reflecting their importance to cell function. They also suggest that proteins within similar functional categories may share similar regulatory transcriptional mechanisms therefore accounting for different functional groups of proteins showing varying levels of correlation with corresponding mRNA levels (Greenbaum *et al.,* 2003; Guo *et al.,* 2008).

There are also methodological factors which may play a part in correlating mRNA and protein abundance. In this present study immunoblotting and immunofluorescence were used to analyse

protein abundance and a commercially available RT^2 Profiler™ PCR Array was used to analyse mRNA levels. It is worth considering that whilst continuous developments are being made to allow for more improved and accurate analysis of gene expression, due to the comparatively complex nature of proteins, analysis of protein abundance is still relatively quite crude. The level of precision of these methods needs to be considered. In this study two separate methodologies were employed to assess protein levels, immunoblotting and immunofluorescence and in addition a semi-quantitative immunohistochemistry analysis was also utilised to further assess changes to XRCC3 protein expression to reinforce the data observed by these two methods. Whatever the inaccuracies of analysing protein expression and judging protein abundance by the methods used, some of the most dramatic changes in transcription observed in this present study (for example, the 6.59 fold decrease in *BRCA1*) were definitely not reflected in protein abundance.

5.9.3 ATR protein expression upon aspirin treatment

Although a distinct down-regulation of ATR protein expression was not observed (Figure 5.1) immunofluorescence analysis of ATR protein expression showed that there was a clear indication of a re-localisation of the protein at a cellular level (Figure 5.2). A subtle redistribution of ATR was apparent in aspirin treated cells, with fluorescence becoming more diffuse in treated cells, possibly reflecting aspirin treatment altering protein translocation/localization. Changes in subcellular localization of ATR following exposure to DNA damaging agents and DNA replication inhibitors have previously been reported (Tibbetts *et al.,* 2000) and suggest that ATR may re-localize within the cell to phosphorylate substrates.

5.9.4 BRCA1 protein expression upon aspirin treatment

Immunoblotting and immunofluorescence analysis of BRCA1 expression in SW480 cells upon 1 mM aspirin treatment for 48 hours, do not suggest a dramatic down-regulation of protein expression in correlation with changes observed in mRNA expression (Figure 5.3 and Figure 5.4). Indeed, it could be considered that there is a slight increase in BRCA1 protein expression upon aspirin treatment seen by immunoblotting (Figure 5.3). This would correspond with the independent quantitative real-time PCR validation analysis which showed a small, non-significant ($P > 0.05$) increase in mRNA expression (Figure 4.21). However, analysis on further samples would need to be carried out to confirm this. This was not possible to carry out in the present study due to limited time available to carry out experiments at KuDOS Pharmaceuticals.

The down-regulation of the tumour suppressor protein BRCA1 upon aspirin treatment is of interest as recently published data indicates that regular NSAID and aspirin intake is associated with an increase in breast cancer (Friss *et al.,* 2008). Immunoblot analysis of BRCA1 does suggest a slight decrease in protein expression in the breast cancer cell line MCF7 when treated with 1 mM aspirin for 48 hours. The three treated samples appear to be less intense than two out of the three untreated samples (Figure 5.5). However, the change in protein expression is not overt, and again further analysis would need to be carried out to confirm any change which was not possible to do due to limited time available at KuDOS Pharmaceuticals. This study does have the potential to be expanded further by examining changes in BRCA1 in MCF7 cells upon treatment with different concentrations of aspirin, specifically those concentrations which cause a significant decrease in MCF7 cell viability (i.e., 5 mM and 10 mM). It would also be interesting to see if an RT^2 Profiler™ PCR Array analysis of DNA damage signalling pathway genes highlighted any potential genes of interest in breast cancer to help elucidate a pathway for the mechanism of aspirin in these cells. It would be interesting to analysis BRCA1 protein expression in MCF7 cells after treatment with a higher concentration of aspirin which does cause a significant decrease in cell viability (such as 5 mM or 10 mM) to see if BRCA1 expression is altered at these concentrations and whether it relates to changes in cell viability.

Interestingly, immunoblot analysis of both SW480 cells and MCF7 cells show a double band at approximately the expected size for BRCA1 (220 kDa). The reason for this double band is unknown although has been previously observed (Personal communication: Charlotte Knights, KuDOS Pharmaceuticals, Cambridge, UK). However, BRCA1 double bands have previously been reported (Coene *et al.,* 2005; Thomas *et al.,* 1997; Scully *et al.,* 1997; Chen *et al.,* 1996). Coene *et al.,* (2005) analysed BRCA1 expression in different fractions of HeLa cells and upon immunoblot analysis two bands were observed for BRCA1 at 200 kDa and 220 kDa which would correspond with the bands observed in this study. The presence of two bands suggest that there may be two forms of the protein and Coene *et al.,* (2005) show that the top band (220 kDa) may be a phosphorylated BRCA1 present within the cell, specifically within the mitochondria, and the presence of a hyperphosphorylated isoform of BRCA1 is supported by other studies (Chen *et al.,* 1996; Ruffner and Verma, 1997; Thomas *et al.,* 1997). Since the protein samples used in this present study were total cell extracts this observation may explain the double band observed.

BRCA1 is known to be expressed in a cell cycle dependent manner (Ruffner and Verma, 1997; Thomas *et al.,* 1997) and this may explain the lack of correlation between mRNA expression and

protein abundance and also the discrepancies seen in protein analysis. As can be seen by immunoblot analysis for both SW480 and MCF7 samples (Figures 5.3 and 5.5), there are differences in band densities within treated and untreated samples, even though there is equal loading of samples. This may suggest that cells were at different stages of the cell cycle and therefore expressing varying levels of BRCA1 and this needs to be taken into consideration.

5.9.5 GADD45α protein expression upon aspirin treatment

The up-regulation of *GADD45α* transcription upon aspirin treatment seen in the present study has been previously reported (Iizaka *et al.,* 2002; Yin *et al.,* 2006) and can therefore be interpreted to act as a positive control validating the RT^2 Profiler™ PCR Array data. However, concordance between *GADD45α* gene transcription and protein expression was not seen in the present study (Figures 5.6 and 5.7). This suggests that conclusions drawn from studies where only *GADD45α* expression at the transcript level has been measured but not demonstrated explicitly at the level of protein expression have to be viewed cautiously. A similar observation was seen for changes in GADD45α protein expression as analysed by immunoblotting and immunofluorescence (Figures 5.6 and 5.7) as was seen for ATR protein expression (Figures 5.1 and 5.2). Although a distinct up-regulation is not observed, immunofluorescence analysis demonstrates a re-localisation of the protein into the nucleus of the cell (Figure 5.7). In 1 mM aspirin treated cells there appeared to be an increase in fluorescence within the nucleus of the cell (as indicated by arrows) compared to untreated cells. This suggests that whilst overall protein expression is not increased, a change to the protein distribution within the cells was occurring.

5.9.6 Differential expression of XRCC3 in colon cancer cell lines upon aspirin treatment

The results presented in this study show the novel finding that XRCC3 protein expression in SW480 cells was increased upon 1 mM aspirin treatment for 48 hours (Figure 5.10). XRCC3 protein expression was increased in SW480 cells upon 1 mM aspirin treatment after 48 hours compared to untreated cells by immunoblotting, immunofluorescence and also by semi-quantitative immunohistochemical analysis. The over-expression of XRCC3 upon aspirin exposure may have implications to the sensitivity of cells to chemotherapeutic agents, particularly in light of the ubiquitous use of aspirin in healthcare. Indeed, previous research in the breast cancer cell line MCF7 demonstrated that over-expression of XRCC3 induced cisplatin resistance (Xu *et al.,* 2005).

In contrast, the MMR-deficient cell line HCT116 showed a decrease in XRCC3 expression upon aspirin treatment. Previous studies have shown that, again in contrast to XRCC3 over-expression and cisplatin resistance, XRCC3 deficient HCT116 cells have increased sensitivity to cisplatin and also mitomycin C (Yoshihara *et al.,* 2004). Also, depletion of XRCC3 by siRNA in MCF7 cells inhibited cell proliferation, leading to accumulation of DNA breaks and triggering activation of p53-dependant cell death suggesting other consequences of loss of XRCC3 (Loignon *et al.,* 2007). Although some studies have shown no association between polymorphisms in XRCC3 and colorectal cancer risk (Mort *et al.,* 2003; Yeh *et al.,* 2005; Tranah *et al.,* 2004; Jin *et al.,* 2005; Skjelbred *et al.,* 2006) some studies have (Improta *et al.,* 2008; Jin *et al.,* 2005) and in addition XRCC3 polymorphisms have also been associated with breast and lung cancer susceptibility (Smith *et al.,* 2003; Jacobsen *et al.,* 2004).

It is important to note that the dsyregulation of XRCC3 protein expression shown in this present study is not necessarily indicative of a direct role of XRCC3 in aspirin mediated cell death. Unlike SW480 cells, the HCT116 cell line is MLH1 deficient and has wild type p53 and the consequence of the difference in MMR status of the two cell lines and the stark contrast in changes of XRCC3 expression upon aspirin treatment should not be overlooked. The p53 status of the two cell lines may be the reason for the differences seen. It has been reported that XRCC3 depletion in MCF7 cells caused p53 dependent cell death and p53 depleted cells were more resistant to cell death caused by XRCC3 depletion (Loignon *et al.,* 2007). This suggests that the mechanisms of action causing cell death following aspirin treatment in the two cell lines may be dependent on p53 status. It may be that a decrease in XRCC3 expression triggers p53 dependent cell death in HCT116 cells. One future key experiment to therefore consider is to investigate changes to XRCC3 expression upon aspirin treatment in the HCT116 p53 -/- cell line. Whether the decrease of XRCC3 is a direct consequence of aspirin or a consequence of other mechanisms involved in aspirin mediated cell death is yet to be elucidated. MLH1 status may also account for the difference in XRCC3 expression observed in the two cell lines. The XRCC3/RAD51C complex is localised to crossover sites in a MLH1-dependent manner (Liu *et al.,* 2006), and it is also known that *MLH1* deficiency reduces crossovers (Baker *et al.,* 1996) which again may explain loss of XRCC3 in the MLH1 deficient cell line HCT116. Goel *et al.,* (2003) showed an increase in MLH1 expression upon aspirin treatment in SW480 cells and this may account for the concomitant increase in XRCC3 shown in this present study.

5.9.7 Conclusion

The results demonstrate via RT^2 Profiler™ PCR Array analysis that treatment with 1 mM aspirin for 48 hours causes decreases in gene expression of *ATR* and *BRCA1* and increases in *XRCC3* and *GADD45α* transcript expression (Table 4.2) in SW480 cells. However, protein expression analysis suggests that changes in protein expression do not necessarily correlate with transcript levels of these genes. This has relevance to microarray studies that solely rely on analysis of message and thus raises the concern of the validity of such studies that do not provide evidence relating to changes at a protein level. This research highlights the importance of testing changes in protein expression to confirm gene expression analysis. It may be that previous microarray research has highlighted changes in mRNA but these changes are not relevant at the protein level and therefore make very little difference in actual disease pathogenesis and/or in cytotoxicity.

More importantly, this research has provided evidence that XRCC3 protein expression is up-regulated upon 1 mM aspirin treatment for 48 hours in MMR-proficient SW480 colorectal cancer (Figures 5.8, 5.9 and 5.10) and down-regulated in MMR-deficient HCT116 colorectal cancer cells (Figure 5.11). The regulation of this key component of homologous recombination repair may be disrupted by aspirin exposure resulting in dysregulation of repair and apoptotic responses with important implications for the response of host cells to chemotherapeutic agents.

Chapter 6

Discussion

6 Discussion

6.1 sMBD4 and MLH1 binding

sMBD4 is a novel alternatively spliced form of the DNA glycosylase MBD4, which to date has no known function. As MLH1 and MBD4 are reported to be transcriptionally coupled (Bellacosa *et al.,* 1999), this study investigated whether sMBD4 and MLH1 were also binding partners with the aim of determining a potential role of sMBD4. Purified, functional sMBD4 was crucial to protein-protein interaction studies. Therefore attempts were made to purify sMBD4 using a novel native purification technique as an alternative method to the Owen *et al*., (2007) denaturing purification method. However, degradation of sMBD4 was greatly increased and protein purified via this native method failed to show any repair activity demonstrating that the protein purified was non-functional which negated the benefits of this purification technique (e.g. Figure 3.5 and Figure 3.10). Therefore, denaturing extraction and refolding of sMBD4 may prove to be a more efficient method for protein recovery and retention of protein activity and this method was used to purify subsequent sMBD4 for protein binding assays.

To determine binding between sMBD4 and MLH1 several interaction assays were attempted as potential methods to investigate binding between the two proteins. These assays were dot blot assays, immunoprecipitation assays and glycosylase activity assays (e.g. Figure 3.13, Figure 3.14 and Figure 3.15). However, the assays used failed to demonstrate a specific protein-protein interaction between the two proteins and it was decided that any further investigation into this would be limited.

Although this study was unsuccessful in determining a specific protein-protein interaction between MLH1 and sMBD4, previous studies utilising yeast-two hybrid systems have suggested an interaction between MLH1 and full length MBD4 (Bellacosa *et al.,* 1999, Macpartlin *et al.,* 2003; Kondo *et al.,* 2005). However, one particular study using tandem affinity purification, found no interaction between the two proteins (Cannavo *et al.,* 2006). It may be that differences in methodology between this study and previous studies account for the differences in findings observed.

6.2 Salicylates and MBD4 gene expression

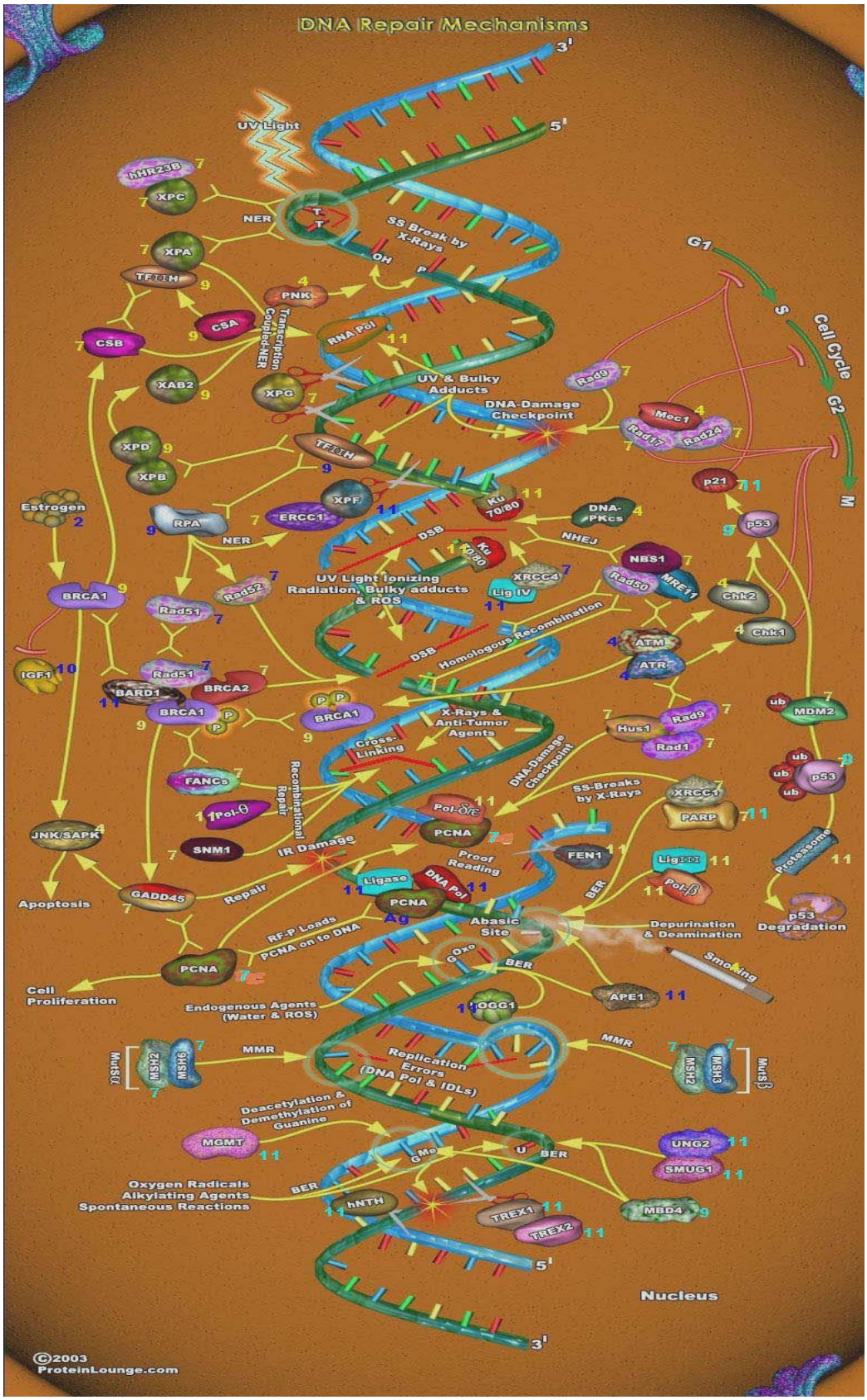
MLH1 has been shown to be over-expressed upon aspirin treatment in colorectal cancer cells (Goel *et al.,* 2003). As MBD4 has been shown in previous studies to be a novel interacting partner of MLH1, the current study investigated whether there was a concominant increase in *MBD4* gene expression upon aspirin treatment utilising semi-quantitative PCR and quantitative real-time PCR. The results presented in this study show that *MBD4* gene expression is not significantly altered upon 1 mM aspirin treatment for 48 hours (Figure 4.16A), but shows evidence for an increase in *MBD4* expression upon treatment with 1 mM salicylic acid for 48 hours (Figure 4.16B).

6.3 Aspirin and DNA repair signalling pathways gene expression

In view of the study carried out by Goel *et al.,* (2003) which suggests a role for the MMR system in the mechanism of aspirin in colorectal cells, it was further investigated whether other DNA damage signalling pathway genes are altered in expression upon aspirin treatment in the colorectal cell line SW480. Interestingly, the results showed that *BRCA1*, *ATR* and *MAPK12* are significantly down-regulated and *XRCC3* and *GADD45α* are up-regulated (Table 4.2).

In addition to the statistically significant changes in gene transcription findings found (Table 4.2), there are other interesting observations. Figure 6.1 shows that ATM/ATR interacts with PCNA through the HUS, RAD1 and RAD9A complex. The results from the PCR array showed that transcription of these genes was decreased by less than 2 fold. Another interesting group interacting with ATM/ATR is the MRE11A, RAD50 and NBS1 (MRN) complex. The array data showed an increase in gene transcription of the genes encoding these proteins. Although all these findings were found to be statistically non-significant, they do highlight that key complexes show similar changes in gene transcription upon aspirin treatment and can give some confidence in the array technique. In addition this shows that a number of different pathways may be affected by aspirin. The MRN complex plays a central role in sensing DNA double strand breaks and recruiting ATM to sites of DNA damage (Lord et al., 2006; Lavin et al., 2006). Changes in expression of these genes could potentially impact this pathway and have a significant effect on DNA repair. These observations therefore raise an important issue of the term 'significant'. Although 'significant' has a strict statistical meaning, this is an example of where statistically non-significant changes could have a possible biological significance.

Figure 6.1 DNA Damage Signalling Pathways (Image taken from www.sabiosciences.com).



6.4 Aspirin and DNA repair signalling pathways and protein expression

Further to the investigation into changes in gene transcription, changes in the subsequent protein expression were also analysed. However, a correlation between BRCA1, ATR and GADD45α gene and protein expression was not observed by immunoblotting and immunofluoresence analysis (Figures 5.1, 5.2, 5.3, 5.4, 5.6 and 5.7). This highlights the lack of correlation between gene transcription and protein abundance and that previous studies carried out which look solely at transcription data and provide no evidence for changes at a protein level must be viewed with caution (Chapter 5). Essentially, it is protein interactions which account for cellular mechanisms and pathway signalling and therefore although gene transcription data can provide an insight as to changes occurring, the effects at a protein level have to be considered.

The present study reports a clear up-regulation of XRCC3 protein expression in SW480 cells in correlation with gene transcription results utilising immunoblotting, immunofluorescence and immunohistochemical techniques (Figures 5.8, 5.9 and 5.10). Further to this, XRCC3 protein expression was analysed in HCT116 cells and conversely to XRCC3 expression upon aspirin treatment in SW480 cells, a down-regulation of XRCC3 protein was observed (Figure 5.11). Aspirin may therefore play a role in dysregulating XRCC3 expression in colorectal cancer cells. These two cells lines differ not only in MMR status and p53 status (see Chapter 4, Table 4.1), but they also differ in COX-2 expression. The significance of altered levels of XRCC3 in these cell lines needs to be considered. These observations would indicate that the responses to DNA damage and repair are likely to be different in these cell lines. In addition, the difference in COX-2 expression would suggest that the metabolism of aspirin in these cells may also be different. These differences may account for the differential expression in XRCC3 observed.

A recent study has shown that XRCC3 gene expression was significantly up-regulated in patients on a flavonoid-rich diet (Guarrera *et al.,* 2007). Flavonoids are a class of polyphenols found in fruit and vegetables. It was hypothesised that flavonoids may play a role in reducing oxidative stress via antioxidant effects such as repair of DNA damage caused by reactive oxygen species by modulating expression of DNA repair genes (Guarrera *et al.,* 2007).

Differential expression of XRCC3 may have significance for treatment as this implies that certain patients may have increased resistance to some chemotherapeutic agents and others may be

more sensitive. Therefore it is possible that a down-regulation of this global repair enzyme will enhance the 'mutator' phenotype/instability of the tumour.

6.5 Significance of Salicylic Acid in Animals and Plants

This present study has shown that salicylic acid significantly up-regulated *MBD4* gene transcription in colorectal cancer cells. Salicylic acid is a naturally occurring salicylate and a metabolite of aspirin. Salicylic acid is a key plant signalling hormone involved in growth regulation, environmental stress responses and host defence against an array of pathogens and the mechanistic pathway of salicylic acid is well understood in plants.

Although of obvious importance in the plant system, salicylic acid has also proven to be beneficial as a therapeutic agent, essentially as an anti-inflammatory prior to the synthesis of aspirin. In addition, colorectal cancer risk has been shown to be reduced in patients with a rich plant based diet. This may be due to the naturally occurring presence of salicylates in fruit, vegetables, herbs and spices. Also, since aspirin is metabolised to salicylic acid in the gut it is reasonable to suggest that some of the anti-tumorgenic effects of aspirin could be due to salicylic acid. It has been speculated that salicylic acid may be a link between aspirin and a high fibre, plant – based diet and a reduced risk of colorectal cancer (Paterson and Lawrence, 2001). Indeed, it has been suggested that differences in diet could account for population differences to cancer risk and incidence and this may be due to differences in the amount of salicylate rich produce consumed (Paterson *et al.,* 2006).

One study found that the total level of salicylates provided by a western diet is about 0 –15 µmol/day and that at this low level the effect on disease risk is negligible (Janssen *et al.,* 1997). However, this study was based on urinary measurements of salicylate and therefore not a necessarily true indicator of presence in the blood, gut lumen or tissue. A subsequent study measured serum salicylic acid concentration and found that salicylic acid is found in higher concentrations in vegetarians than non-vegetarians and in addition, that there is overlap between the serum concentrations of salicylic acid in vegetarians and patients taking a low dose aspirin (Blacklock *et al.,* 2001). Moreover, it has been shown that salicylic acid is present in the serum of individuals not taking any salicylate based drugs (Paterson *et al.,* 1998). These findings support the idea that salicylic acid is not only present in the serum of individuals due to intake of salicylate drugs, but is also present endogenously through diet and may play a role in the protective effect

against colorectal cancer. Most intriguingly Paterson *et al.,* (2008) found that salicylic acid blood concentrations were higher in some carnivorous animals (for example the burrowing owl) than in herbivores. When the gut bacteria were killed with antibiotics in mice, they could still detect salicylic acid in blood. This interestingly suggests that salicylic acid could be endogenously produced in humans.

Salicylic acid has been shown to modulate oxidative stress and glutathione peroxidise activity in the rat colon (Drew *et al.,* 2005). Glutathione peroxidises are involved in the COX-2 pathway regulating the formation of prostaglandins. In rat models with an increase in oxidative stress and prostaglandin production (thought to be due to a decrease in glutathione peroxidise), salicylic acid supplementation of the diet caused an increase in glutathione peroxidise and a subsequent decrease in both oxidative stress and the formation of prostaglandins (Drew *et al.,* 2005). This is an important observation as it suggests that salicylic acid, like aspirin, may interact with the COX pathway to modulate prostaglandin production.

One interesting hypothesis is that the signalling pathway for salicylic acid may be in effect operating in a similar way in animals (Garreton *et al.,* 2002). In plants, salicylic acid is involved in coordinating systemic acquired resistance (SAR) in response to pathogens. Salicylic acid accumulates and these elevated levels lead to redox status changes which activates SAR. This change in redox status causes the non-expressor of pathogenesis-related genes1/no immunity 1 (NPR1/NIM1) protein to translocate from the cytosol to the nucleus which in turn leads to the subsequent activation of SAR. It is been shown that key NPR1 interacting partners are WRKY and TGA transcription factors. The MAPK signalling pathway is common to all eukaryotes and interestingly, NPR1/NIM1 shows homology to IκBα a key component of the NFκB signalling pathway (Ryals *et al.,* 1997). It could be that aspirin and salicylates cause IκBα to move to the nucleus in human cells as it does with plant cells and this may be causing the gene transcription implications associated with aspirin treatment. Indeed, this is supported by Stark *et al.,* (2001; 2006) and Din *et al.,* (2004; 2005) who propose that IκBα is degraded and shows nuclear translocation upon aspirin treatment in colorectal cancer cells. Wang *et al.,* 2006 show by array work in Arabidopsis that key NPR1 interacting proteins are WRKY transcription factors. It is interesting to note that although WRKY transcription factors are plant specific (Wang *et al.,* 2006), TGA transcription factors are found in all eukaryotes. Garreton *et al.,* 2002, interestingly makes the link between salicylic acid responsive element in plants and AP-1 box in animals (TGACTAT)

and TGA factors share homology with c-jun. This makes oxidative stress response the key common link.

6.6 Aspirin dosage

One key question raised by this study is of the dosage of aspirin utilised and its potential effects. There is a lack of consistency in the literature regarding dosages utilised for both clinical and experimental studies. Clinical trials have shown that the low dose aspirin (81mg) is more protective against colorectal cancer than the high dose aspirin (325mg) (Baron *et al.,* 2003). In addition, experimental studies have shown that different dosages of aspirin can have different effects. Hardwick *et al.,* (2004) showed that different concentrations of aspirin have different effects on gene expression levels. This lack of consistency, especially with regards to what dose is clinically relevant makes it difficult to decide on appropriate drug concentrations for experimental work. For this study 1 mM aspirin and salicylic acid was utilised as, in addition to it being reported to be physiologically relevant (Stark *et al.,* 2001), results in this present study found that this was the lowest concentration of aspirin which caused significant cell death as observed by cell viability assays. In addition, this concentration was utilised in the study analysing the effects on DNA repair proteins (Goel *et al.,* 2003) which is a basis for this present study. However, it is worth considering that although effects may not be seen on cell viability by using lower doses of aspirin and salicylic acid, there may be subtle changes occurring to transcription levels which may result in significant alterations at a protein level. This is not only a consideration for aspirin given as treatment but also a point to consider about endogenously present salicylic acid. The definition of a 'significant' dose of aspirin/salicylic acid may then vary dependent upon its effect as an analgesic or, at potentially lower doses, as a regulator of gene expression.

Crucially, endogenous salicylic acid present in the gut could be causing subtle changes continuously. It could be speculated that the reason why there is conflict as to whether aspirin reduces the risk of certain cancers, (including colorectal cancer), is that each individual has a different gene expression profile along with a unique amount of endogenous salicylate present. Each individual could potentially have a different genetic response to salicylate exposure and there may even be different classes of people who have evolved different responses to salicylic acid and hence have different genetic responses to salicylate exposure. Further to this, it would be interesting to determine salicylic acid levels specifically in the gut. Most studies have analysed plasma salicylate levels and these values have been utilised for subsequent studies as an indicator of endogenous salicylate levels. However, one interesting hypothesis is that the gut may be

exposed to different levels of salicylate compared to plasma levels measured on account of salicylate in the diet presumably from plant material. Therefore it may be that the cells in the gut have evolved in response to salicylate based on exposure to plant material, specifically in the metabolism of these compounds and this may be a factor worth considering especially as aspirin has been shown to be cytotoxic to colorectal cells in particular. It may be that salicylates are acting as signalling molecules in all eukaryotes (Paterson *et al.,* 2008), achieving subtle transcriptional effects at low concentrations. The anti-tumourgenesis and anti-inflammatory effects could possibly be artefacts of high doses.

6.7 Future work

This study failed to demonstrate a specific protein – protein interaction between sMBD4 and MLH1, possibly due to the methodologies utilised and so this does not necessarily mean that an interaction between these two proteins does not occur. It would be useful to try alternative methods to determine an interaction such as Biacore analysis using purified proteins.

To further characterise *MBD4* gene expression upon exposure to salicylates it would be interesting to determine if salicylates cause alterations of *MBD4* gene transcription in other cancer cell lines. In addition, protein expression of MBD4 upon salicylate exposure would be interesting to determine, in particular the localisation of MBD4 and whether it is related to the localisation of MLH1 upon aspirin exposure.

It would be interesting to carry out RT^2 Profiler™ PCR array studies to analyse any gene transcription changes of the DNA damage signalling pathway genes utilising lower doses of aspirin and salicylic acid. It is possible that low doses of salicylates may have subtle transcriptional effects but they may be of significant consequence at a protein level especially with regard to DNA repair enzymes. Changes in expression of repair enzymes will have a direct consequence on repair of DNA lesions, therefore even a small difference in gene and/or protein expression could alter the number of DNA lesions being repaired and this will impact the cell significantly. Therefore even though change in expression of these enzymes may be considered small or negligible, the consequence on cell mechanisms such as DNA repair may be biologically significant.

In addition, it would also be of interest to analyse changes in gene and protein expression, especially of XRCC3, at different time intervals. In this study, 48 hours was chosen as this was the time at which cell death was seen to occur. However, it would be interesting to know how quickly an increase in gene and protein expression is seen after aspirin treatment. In addition, one key study is to determine XRCC3 expression in a panel of colorectal cancer cell lines to further analyse the differential response observed in this study.

To help further understand the effects of aspirin on XRCC3 expression and to clarify a potential pathway of action, XRCC3 deficient and XRCC3 proficient cell lines could be treated with aspirin to see if any differences to aspirin exposure occur.

The direct effect of aspirin on DNA damage should also be further considered. The finding that aspirin induces expression of DNA repair enzymes (Chapter 5) might suggest that aspirin is having a damaging effect on DNA. Indeed, many chemotherapeutic drugs are cytotoxic based on their ability to damage DNA (Madhusudan and Hickson, 2005). It could be that as aspirin is metabolised, the acetyl groups which are released modify cellular proteins and also DNA and this may in part explain why aspirin would affect DNA MMR proteins. The findings from the present study show that aspirin modulates XRCC3 expression in two different colorectal cancer cell lines and therefore it could be argued that aspirin is in essence inhibiting a DNA repair pathway in patients based on their genetic profile.

The significant changes in XRCC3 expression upon aspirin treatment is also worth further exploring. In the broader context of cancer therapy, BER has been considered as a potential target for the development of novel treatments (Sharma and Dianov, 2007). Essentially, dsyregulation of BER proteins can contribute to carcinogenesis. However, a down-regulation of certain proteins may impair BER and lead to an accumulation of DNA damage resulting in subsequent genomic instability and apoptosis. Therefore understanding the pathway and its modifications as a result of drug treatment is essential (Sharma and Dianov, 2007). This is highlighted in this study where aspirin treatment was found to effect the expression of XRCC3 in two different colorectal cancer cells. This may be a direct result of two different pathways involved in these cells as they differ in MMR status. It is interesting that cancers of the same class appear to have variation gene expression and therefore of signalling pathways. This again is an important consideration for the impact of chemotherapeutic agents (Copeland and Jenkins, 2009).

6.8 Conclusion

This study has shown that MBD4 gene expression is up-regulated upon 1 mM salicylic acid treatment for 48 hours. In addition, this study demonstrates via RT^2 Profiler array analysis that aspirin causes a decrease in *ATR, BRCA1* and *MAPK12* and increases in *XRCC3* and *GADD45α* gene transcription. Protein expression analysis suggests that changes in transcript levels of these genes do not necessarily correlate with changes in protein expression. This finding has relevance to microarray studies that solely rely on analysis of message and thus raises the concern of the validity of such studies that do not provide evidence of changes at a protein level. However, XRCC3 expression is significantly up-regulated as both transcript and protein in the SW480 cancer cell line and as a result of exposure to aspirin and XRCC3 protein expression was found to be down-regulated in the HCT116 cancer cell line. Aspirin may have the capacity to dysregulate XRCC3 expression and thus function, with important implications for the response of host cells to chemotherapeutic agents.

Appendix

Table A1. Gene Table of genes analysed by RT Profiler™ PCR array

Position	uniGene	GenBank	Symbol	Description
A01	Hs. 431048	NM_005157	*ABL1*	V-abl Abelson murine leukemia viral oncogene homolog 1
A02	Hs.601206	NM_198889	*ANKRD17*	Ankyrin repeat domain 17
A03	Hs.73722	NM_080649	*APEX1*	APEX nuclease (multifunctional DNA repair enzyme) 1
A04	Hs.367437	NM_000051	*ATM*	Ataxia telangiectasia mutated (included complementation groups A, C and D)
A05	Hs.271791	NM_001184	*ATR*	Ataxia telangiectasia and Rad3 related
A06	Hs.533526	NM_000489	*ATRX*	Alpha thalassemia/mental retardation syndrome X-linked (RAD54 homolog, *Saccharomyces cerevisiae*)
A07	Hs.194143	NM_007294	*BRCA1*	Breast cancer 1, early onset
A08	Hs.519162	NM_006763	*BTG2*	BTG family, member 2
A09	Hs.292524	NM_001239	*CCNH*	Cyclin H
A10	Hs.184298	NM_001799	*CDK7*	Cyclin-dependent kinase 7 (MO15 homolog, *Xenopus laevis,* cdk-activating kinase)
A11	Hs.24529	NM_001274	*CHEK1*	CHK1 checkpoint homolog (*Schizosaccharomyces pombe*)
A12	Hs.291363	NM_007194	*CHEK2*	CHK2 checkpoint homolog (*Schizosaccharomyces pombe*)
B01	Hs.135471	NM_006384	*CIB1*	Calcium and integrin binding (calmyrin)
B02	Hs.249149	NM_001279	*CIDEA*	Cell death-inducing DFFA-like effector a
B03	Hs.151573	NM_004075	*CRY1*	Cryptochrome 1 (photolyase-like)
B04	Hs.290758	NM_001923	*DDB1*	Damage-specific DNA binding protein 1, 127kDa

B05	Hs.505777	NM_004083	*DDIT3*	DNA-damage-inducible transcript 3
B06	Hs.339396	NM_007068	*DMC1*	DMC1 dosage suppressor of mck1 homolog, meiosis-specific homologous recombination (yeast)
B07	Hs.435981	NM_001983	*ERCC1*	Excision repair cross-complementing rodent repair deficiency, complementation group 1 (includes overlapping antisense sequence)
B08	Hs.487294	NM_000400	*ERCC2*	Excision repair cross-complementing rodent repair deficiency, complementation group 2 (xeroderma pigmentosum D)
B09	Hs.498248	NM_130398	*EXO1*	Exonuclease 1
B10	Hs.591084	NM_004629	*FANCG*	Fanconi anemia, complementation group G
B11	Hs.409065	NM_004111	*FEN1*	Flap structure-specific endonuclease 1
B12	Hs.292493	NM_001469	*XRCC6*	X-ray repair complementing defective repair in Chinese hamster cells 6 (Ku autoantigen)
C01	Hs.80409	NM_001924	*GADD45A*	Growth arrest and DNA-damage-inducible, alpha
C02	Hs.9701	NM_006705	*GADD45G*	Growth arrest and DNA-damage-inducible, gamma
C03	Hs.545196	NM_002066	*GML*	GPI anchored molecule like protein
C04	Hs.577202	NM_005316	*GTF2H1*	General transcription factor IIH, polypeptide 1, 62kDa
C05	Hs.191356	NM_001515	*GTF2H2*	General transcription factor IIH, polypeptide 2, 44kDa
C06	Hs.386189	NM_016426	*GTSE1*	G-2 and S-phase expressed 1
C07	Hs.152983	NM_004507	*HUS1*	HUS1 checkpoint homolog (*Schizosaccharomyces pombe*)
C08	Hs.503048	NM_002180	*IGHMBP2*	Immunoglobulin mu binding protein 2

C09	Hs.17253	NM_054111	*IHPK3*	Inositol hexaphosphate kinase 3
C10	Hs.61188	NM_033276	*XRCC6BP1*	XRCC6 binding protein 1
C11	Hs.1770	NM_000234	*LIG1*	Ligase 1, DNA, ATP-dependent
C12	Hs.463978	NM_002758	*MAP2K6*	Mitogen-activated protein kinase kinase 6
D01	Hs.432642	NM_002969	*MAPK12*	Mitogen-activated protein kinase 12
D02	Hs.35947	NM_003925	*MBD4*	Methyl-CpG binding domain protein 4
D03	Hs.195364	NM_000249	*MLH1*	MutL homolog 1, colon cancer, nonpolyposis type 2 (*Escherichia coli*)
D04	Hs.436650	NM_014381	*MLH3*	MutL homolog 3 (*Escherichia coli*)
D05	Hs.509523	NM_002431	*MNAT1*	*Ménage a trois* homolog 1, cyclin H assembly factor (*Xenopus laevis*)
D06	Hs.459596	NM_002434	*MPG*	N-methylpurine-DNA glycosylase
D07	Hs.192649	NM_005590	*MRE11A*	MRE11 meiotic recombination 11 homolog A (*Saccharomyces cerevisiae*)
D08	Hs.597656	NM_000251	*MSH2*	MutS homolog 2, colon cancer, nonpolyposis type 1 (*Escherichia coli*)
D09	Hs.280987	NM_002439	*MSH3*	MutS homolog 3 (*Escherichia coli*)
D10	Hs.271353	NM_012222	*MUTYH*	MutY homolog (*Escherichia coli*)
D11	Hs.396494	NM_018177	*N4BP2*	Nedd4 binding protein 2
D12	Hs.492208	NM_002485	*NBN*	Nibrin
E01	Hs.66196	NM_002528	*NTHL1*	Nth endonuclease III-like 1 (*Escherichia coli*)
E02	Hs.380271	NM_002542	*OGG1*	8-oxoguanine DNA glycosylase
E03	Hs.20930	NM_020418	*PCBP4*	Poly(rC) binding protein 4
E04	Hs.147433	NM_182649	*PCNA*	Proliferating cell nuclear antigen
E05	Hs.424932	NM_004208	*AIFM1*	Apoptosis-inducing factor, mitochondrion-associated, 1

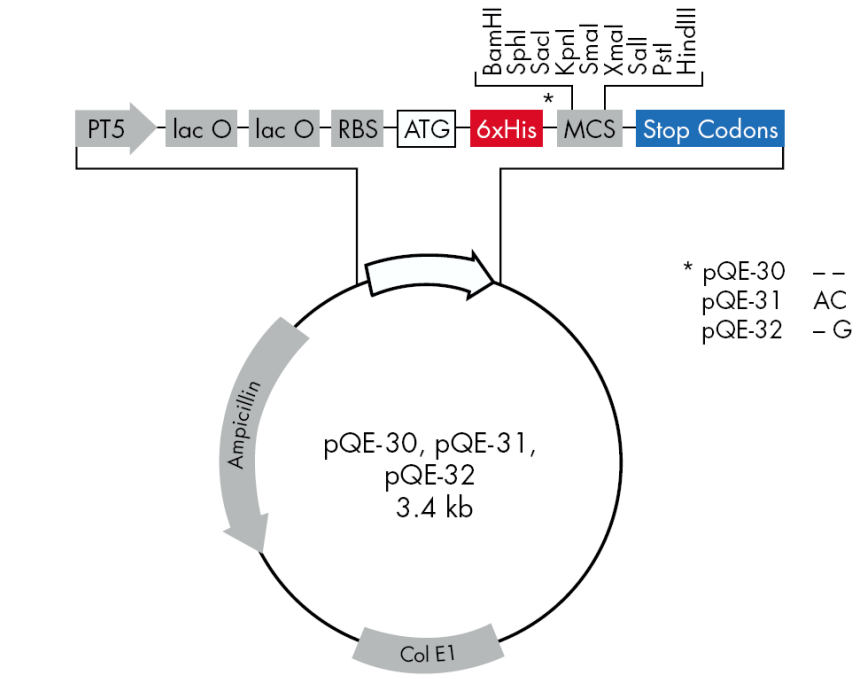
E06	Hs.111749	NM_000534	*PMS1*	PMS1 postmeiotic segregation increased 1 (*Saccharomyces cerevisiae*)
E07	Hs.632637	NM_000535	*PMS2*	PMS2 postmeiotic segregation increased 2 (*Saccharomyces cerevisiae*)
E08	Hs.225784	NM_005395	*PMS2L3*	Postmeiotic segregation increased 2-like 3
E09	Hs.78016	NM_007254	*PNKP*	Polynucleotide kinase 3'-phosphatase
E10	Hs.631593	NM_014330	*PPP1R15A*	Protein phosphatase 1, regulatory (inhibitor) subunit 15A
E11	Hs.491682	NM_006904	*PRKDC*	Protein kinase, DNA-activated, catalytic polypeptide
E12	Hs.531879	NM_002853	*RAD1*	RAD1 homolog (*Schizosaccharomyces pombe*)
F01	Hs.16184	NM_002873	*RAD17*	RAD17 homolog (*Schizosaccharomyces pombe*)
F02	Hs.375684	NM_020165	*RAD18*	RAD18 homolog (*Saccharomyces cerevisiae*)
F03	Hs.81848	NM_006265	*RAD21*	RAD21 homolog (*Schizosaccharomyces pombe*)
F04	Hs.128904	NM_005732	*RAD50*	RAD50 homolog (*Saccharomyces cerevisiae*)
F05	Hs.631709	NM_002875	*RAD51*	RAD51 homolog (RecA homolog, *Escherichia. coli*) (*Saccharomyces cerevisiae*)
F06	Hs.172587	NM_133509	*RAD51L1*	RAD51-like (*Saccharomyces cerevisiae*)
F07	Hs.240457	NM_004584	*RAD9A*	RAD9 homolog A (*Schizosaccharomyces pombe*)
F08	Hs.546282	NM_002894	*RBBP8*	Retinoblastoma binding protein 8
F09	Hs.443077	NM_016316	*REV1*	REV1 homolog (*Saccharomyces cerevisiae*)
F10	Hs.461925	NM_002945	*RPA1*	Replication protein A1, 70kDa
F11	Hs.408846	NM_022367	*SEMA4A*	Sema domain, immunoglobulin domain (Ig), transmembrane domain (TM) and short cytoplasmic domain, (semaphoring) 4A
F12	Hs.591336	NM_014454	*SESN1*	Sestrin 1

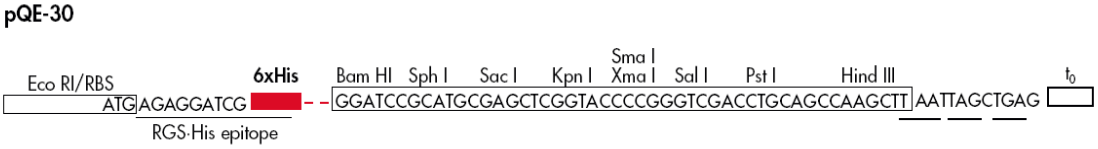
G01	Hs.211602	NM_006306	*SMC1A*	Structural maintenance of chromosomes 1A
G02	Hs.81424	NM_003352	*SUMO1*	SMT3 suppressor of *mif* two 3 homolog 1 (*Saccharomyces cerevisiae*)
G03	Hs.408312	NM_000546	*TP53*	Tumor protein p53 (Li-Fraumeni syndrome)
G04	Hs.192132	NM_005427	*TP73*	Tumor protein p73
G05	Hs.344812	NM_016381	*TREX1*	Three prime repair exonuclease 1
G06	Hs.191334	NM_003362	*UNG*	Uracil-DNA glycosylase
G07	Hs.591907	NM_000380	*XPA*	Xeroderma pigmentosum, complementation group A
G08	Hs.475538	NM_004628	*XPC*	Xeroderma pigmentosum, complementation group C
G09	Hs.98493	NM_006297	*XRCC1*	X-ray repair complementing defective repair in Chinese hamster cells 1
G10	Hs.647093	NM_005431	*XRCC2*	X-ray repair complementing defective repair in Chinese hamster cells 2
G11	Hs.592325	NM_005432	*XRCC3*	X-ray repair complementing defective repair in Chinese hamster cells 3
G12	Hs.444451	NM_016653	*ZAK*	Sterile alpha motif and leucine zipper containing kinase AZK
H01	Hs.534255	NM_004048	*B2M*	Beta-2-microglobulin
H02	Hs.412707	NM_000194	*HPRT1*	Hypoxanthine phosphoribosyltransferase 1 (Lesch-Nyhan syndrome)
H03	Hs.546356	NM_012423	*RPL13A*	Ribosomal protein L13a
H04	Hs.544577	NM_002046	*GAPDH*	Glyceraldehyde-3-phosphate dehydrogenase
H05	Hs.520640	NM_001101	*ACTB*	Actin, beta
H06	N/A	SA_00105	HGDC	Human Genomic DNA Contamination
H07	N/A	SA_00104	RTC	Reverse Transcription Control

H08	N/A	SA_00104	RTC	Reverse Transcription Control
H09	N/A	SA_00104	RTC	Reverse Transcription Control
H10	N/A	SA_00103	PPC	Positive PCR Control
H11	N/A	SA_00103	PPC	Positive PCR Control
H12	N/A	SA_00103	PPC	Positive PCR Contol

Figure A1. pQE30 map and sequence (Taken from Qiagen website: http://www1.qiagen.com/literature/pqesequences/pqe3x.pdf)

Positions of elements in bases	pQE-30
Vector size (bp)	3461
Start of numbering of *Xho*1 (CTCGAG)	1 - 6
T5 promoter/lac operator element	7 - 87
T5 transcription start	61
6 x His-tag coding sequence	127 - 144
Multiple cloning site	145 - 192
Lambda t_0 transcriptional termination region	208 - 302
***rrnB* T1 transcriptional termination region**	1064 - 1162
ColE1 origin of replication	1638
β -lactamase coding sequence	3256 - 2396





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